The Preparation and Properties of the Multiple Forms of Pig Brain Acetylcholinesterase.

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THE PREPARATION AND PROPERTIES OF THE

MULTIPLE FORMS OF PIG BRAIN

ACETYLCHOLINESTERASE

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ABSTRACT

A number of solubilization protocols have been studied with a view to obtaining a suitable preparation of pig brain acetylcholinesterase. The non-ionic detergent Triton X-100 released the greatest amount of enzyme from the membranes whereas extraction with low ionic strength buffer, E.D.T.A, E.G.T.A., trypsin or autolysis were far less successful. Solubilization with Triton X-100 was therefore used in subsequent investigations.

Affinity chromotography was found to give a high degree of purification. Three different ligands which had already been used by other workers in the purification of acetylcholinesterase from other sources were investigated. The most efficient purification was obtained when the enzyme was eluted from a column containing the acetylcholinesterase inhibitor 1-methyl-9-[N$^{\beta}$-(ε-aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide covalently bound to Sepharose 4B. The elution profile also depended on the presence of Triton X-100, the ionic strength and the inhibitor used to remove the enzyme from the column.

A comparative study was made of the multiple molecular forms of acetylcholinesterase obtained by the different solubilization methods and the purified enzyme. The techniques employed included starch block electrophoresis, polyacrylamide gel electrophoresis and density gradient centrifugation. The presence of Triton X-100 throughout the systems was critical to the resolution of the multiple molecular forms. These studies suggest that acetylcholinesterase exists as a monomer with an approximate molecular weight of 68,000 or 83,000 and that the higher molecular weight species are aggregates of these subunits.

The importance of the membrane to the properties of acetylcholinesterase was demonstrated from studies made on the membrane bound and solubilized enzyme and also on the enzyme bound to liposomes. Whereas the enzyme bound to the native membrane or artificial membrane (liposome) gave a break in the Arrhenius plot, the solubilized enzyme did not show this phenomenon.

CONTENTS

ABBREVIATIONS

AChE	=	Acetylcholinesterase	EC 3.1.1.7.
AChR	=	Acetylcholine receptor	
BEU	=	Basic excitation unit	
ChE	=	Cholinesterase	EC 3.1.1.8.
DFP	=	Diisopropylphosphofluoridate	
DTNB	=	5,5′ -dithiobis-(2-nitrobenzoic acid)	
MA	=	Myristic acid	
MAC	=	1-Methyl-9-[N$^{\beta}$ - ε -aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide	
MAP	=	N-Methyl-3-aminopyridinium iodide	
2-PAM	=	2-Pyridine aldoxime methiodide	
PC	=	Egg-phosphatidylcholine	
PTA	=	p-Aminophenyl trimethylammonium bromide hydrobromide	
S	=	Acetylcholine storage protein	

SECTION I: INTRODUCTION

I. INTRODUCTION

1. General Background

A. History

It was in 1913 that Henry Dale first demonstrated that a particular ergot extract when injected intravenously into cats produced a rapid but transient fall in blood pressure. This observation, now documented in Dale's (1914) classic paper, marked the beginning of the history of research into acetylcholine, and the proteinsof the acetyl choline cycle.

In 1918, Führer, while working with leech muscle, showed that physostigmine potentiated the stimulating action of acetylcholine by one million fold, whereas the effect on choline remained unchanged. Dale (1914) had predicted the possibility that acetylcholine arising in the body might be so rapidly hydrolysed by the tissues as to avoid detection, and when Abderhalden & Paffrath (1925) showed that an enzyme catalysing the hydrolysis of acetylcholine was present in the pig intestine, Dale's forecast seemed to be confirmed. Further evidence of an enzyme responsible for hydrolysing acetylcholine was provided the next year by Loewi & Navratil (1926), who demonstrated the cholinesterase-inhibiting properties of physostigmine.

B. Definition and Specificity

Stedman, Stedman & Easson (1932) were the first to ask whether cholinesterases were specific for the breakdown of acetylcholine. Establishment of this fact would have reinforced the idea that the enzyme's physiological function was to hydrolyse the ester and thus inactivate it. Working with horse serum, they found that the esterase present was specific for cholinesters but hydrolysed butyrylcholine faster than acetylcholine. However, Vahlquist (1935) showed that cholinesters were not hydrolysed exclusively by human plasma, and Glick (1938, 1939, 1941) listed several esters which were broken down by the serum enzyme.

Alles & Hawes (1940) made a significant discovery when they found that the cholinesterase of the erythrocyte membrane differed markedly from that of serum in that it had an optimum substrate concentration. Excess substrate inhibited the enzyme and also, acetyl -β- methylcholine was hydrolysed by the red cell enzyme but not by the serum esterase. Richter & Croft (1942) confirmed the differences between the two types of cholinesterase in blood by testing their properties with specific inhibitors and Zeller & Bissegger (1943) showed that the cholinesterase present in brain was similar to that in the erythrocyte.

Nachmansohn & Rothenberg (1944, 1945) while studying the enzyme in many types of conducting tissue showed that the behaviour of the enzyme towards various substrates was very similar in erythrocytes and conducting tissue but quite different to the serum enzyme. The rate of hydrolysis of cholinesters by serum cholinesterase increased with the size of acyl group as follows: acetyl < propionyl < butyryl whereas the turnover number of cholinesterase in conducting tissue fell sharply for acyl groups larger than propionyl. Also, the nature of the alcoholic group was found to be more critical for the activity of the serum cholinesterase than for that of conducting tissue (Augustinsson, K-B.1963; Wilson, 1954). It was therefore apparent that there were fundamental differences between various esterases from different sources and attempts were made to assign a meaningful classification to the enzymes. Mendel & Rudney (1943) coined the terms "true" and "pseudo" cholinesterase to describe the enzymes from erythrocytes and serum respectively, but their classification was based on data which differed from that of other laboratories. Nachmansohn & Rothenberg (1944;1945) classified the enzymes on whether the source was erythrocyte (specific cholinesterase) or nervous tissue (acetylcholinesterase). However, these definitions are very general and Nachmansohn and Rothenberg themselves pointed out that although there was an esterase in erythrocytes which was specific for acetylcholine, the specificity was relative and unconditional. Adams (1949) also criticised the use of the term specificity

because of its implication of the absolute. It therefore became necessary to define the various cholinesterases and also to distinguish them from other esterases. Therefore the Enzyme Commission of the International Union of Biochemistry (1964) classified the cholinesterase into two groups: those which hydrolyse acetylcholine most rapidly and those which hydrolyse propionylcholine or butyrylcholine most rapidly. The former esterase was designated acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7) and the latter as cholinesterase (acylcholine acyl hydrolase EC 3.1.1.8). However, even within the Enzyme Commission's classification of acetylcholinesterase there can be anomolies. For example, the acetylcholinesterase from electric eel electroplax is not inhibited by phenylmethanesulphonyl fluoride (Fahrney & Gold, 1963) but the enzyme of bovine erythrocytes is inhibited by this compound. (Turini, Kurooka, Steer, Carbascio & Singer, 1969).

Throughout the rest of this thesis acetylcholinesterase will be abbreviated to AChE and cholinesterase to ChE. Cholinesterases will be used as a general term for both enzymes (McIntosh 1973). Table I.1 summarises the properties of AChE and ChE.

The Enzyme Commission (1964) also rationalized the classification of other esterases so that B-esterase became carboxylic ester hydrolase (EC 3.1.1.1); A-esterase became aryl ester hydrolase (EC 3.1.1.2), and C-esterase became acetic ester acetyl-hydrolase (EC 3.1.1.6).

C.Occurrence

i. Species distribution. Prosser (1946) and Augustinsson (1948) both showed that a very wide range of organisms possessed the ability to hydrolyse acetylcholine. In fact the ubiquity of AChE is demonstrated by its presence from the lowest groups of the animal kingdom such as the sea anemones, right through to the highest group, the mammals. The snail, Helix pomatia (Augustinsson 1946), the ciliated mononucleated organism Tetrahymena geleii S. (Seaman & Houlihan, 1951) and the sea anemone, Sagartia (Augustinsson, 1948) all show acetylcholine hydrolysing activity.

Table I.1

Specificity of Cholinesterases

	Acetylcholinesterase E.C.3.1.1.7 (AChE)	Cholinesterase E.C.3.1.1.8 (ChE)
Optimal substrate	acetylcholine	(butyrylcholine propionyl or benzoyl choline for some enzymes)
Excess substrate	inhibition	occasional inhibition with cholinesters of aromatic acids
acetyl-β-methyl-choline	substrate	non substrate
butyryl or benzoyl-choline	non substrates	substrates
B.W.62c47	potent inhibitor	weak inhibitor
ethopropazine	weak inhibitor	potent inhibitor
optimum pH	7·5 - 8·0	8·5
distribution	electric organ, erythrocytes, nerve tissue, thymus	serum, pancreas, liver, heart.

Generally however, the highest concentrations of AChE are found within mammals. The exception to this is the very high level of AChE found in the electroplax of electric fish such as Electrophorus electricus and Torpedo marmorata (Marnay, 1937) and some snake venoms (Iyengar, Sehra, Mukerji, Chopra, 1938).

The plant kingdom also contains enzymes which catalyse the hydrolysis of acetylcholine. AChE (Jaffe, 1970) and ChE (Jaffe, 1973) have both been identified in the mung bean. Fluck and Jaffe (1974) published a survey of 60 plant species containing AChE which included the potato and tomato. Kingsbury & Masters (1970) have also put forward a tentative scheme to cover the evolution of proteins with esterase activity through the species' from a common primitive esterase. (reviewed by Masters & Holmes, 1974).

ii. Tissue localization. When Dale discovered that ACh was a neurohumoral transmitter it became apparent that the means of removing it from synapses or neuromuscular junctions was critical to allow further transmission. AChE was found to fit the role of inactivating the transmitter, but the question had to be asked whether the concentration of AChE in nervous tissue was sufficient to hydrolyse the ACh in the necessarily short time span. Various techniques were therefore used to determine the localization of the enzyme, principally centrifugation coupled with enzyme assay or histochemistry coupled with the light or electron microscope.

AChE has proved to be present in nerve fibres synapsing with skeletal muscle, preganglionic and postganglionic fibres in the parasympathetic autonomic nervous system, preganglionic fibres in the sympathetic autonomic nervous system and some central nervous system fibres.

a. Neuromuscular system. Marnay & Nachmansohn (1937, 1938) first showed areas of the frog sartorius which were innervated by motor nerves had an AChE activity up to six times higher than areas which were free from nerve endings. This finding was significant in that it suggested that ACh might have a special role at the myoneural junction - perhaps as a neurohumoral transmitter. Knowing that the volume formed by the nerve endings at the

myoneural junction was small, the actual concentration of enzyme appeared to be remarkably high. However, the reason for this was later discovered when it was found that the synaptic cleft between nerve and muscle was really a mass of infoldings which allowed a much higher surface area to be presented for the hydrolysis of ACh. than originally suspected. Karnovsky (1961), when studying the sarcoplasmic surface of rat skeletal muscle fibres, showed conclusively that AChE was present in high concentrations along the postjunctional membrane and it was suggested in this paper that the enzyme was synthesized locally. It was also shown by Couteaux (1955) and Salpeter (1967) that AChE is not confined to the end plate but is also present throughout the muscle fibres. Friedenberg & Seligman (1972) drew attention however to the dangers in interpreting histochemical data where an enzyme stain has been used. He quotes particularly the work of Adams, Bayliss & Grant (1969) working on nodes of Ranvier where it is argued that local staining in these regions might be due to the actual presence of AChE or alternatively the precipitation of thiocholine by copper in the stain binding to nodal acidic mucosubstances. This issue becomes particularly important when one looks into the problem of whether axonal transmission of the impulse is electrical or chemical in action, (see this thesis).

However, establishment of the fact that AChE is concentrated at the myoneural junction is consistent with Nachmansohn's (1970) neurohumoral transmission theory in which he argues that ACh release is intracellular causing permeability changes in the presynaptic and postsynaptic membranes rather than ACh actually crossing the postsynaptic membrane (see also this thesis).

b. Autonomic nervous system. When it was found that AChE totally disappeared following preganglionic denervation, it was concluded that the enzyme was confined exclusively to the presynaptic terminals (Koelle & Koelle, 1959). The hypothesis was therefore mooted that ACh released in preganglionic synapsis at the presynaptic site, amplifies further ACh

release by a positive feedback mechanism, and this is modulated by the AChE (Koelle, 1962). This fits in with the theory (Burn & Rand, 1959) that ACh facilitates the release of other transmitters at some non-cholinergic nerve endings and might explain the presence of high levels of AChE in parts of the adrenergic nervous system (Koelle, 1971).

However, more recently, by using an improved histochemical method (Koelle, Davis, Smyrl, Fine, 1974) the limitation of AChE to the pre-synaptic membrane has been questioned (Koelle, Davis, Koelle, Smyrl, Fine, 1975), Electron microscopic studies of the cat superior cervical ganglion clearly showed that AChE was present at both the presynaptic and postsynaptic membranes, smooth endoplasmic reticulum and Schwann cell envelope. Koelle et al (1975) therefore think that the reason for the disappearance of the ganglionic AChE consequent upon preganglionic denervation is due to the loss of a trophic factor which maintains the AChE at the postsynaptic membrane as well as the atrophy of the presynaptic membranes themselves.

c. Central nervous system. Sjöstrand (1938) first demonstrated that perfusion of the rat brain cortex with ACh caused a change in the electroencephalogram thus showing the probability of cholinergic mechanisms being involved in the central nervous system. Since then, ACh has been found in the central nervous system of all vertebrates studied, (Nistri, De Bellis, Cammelli, 1975). AChE was shown to be present in frog brain by Shen, Greenfield, Boell,(1955). Since then much research has gone into elucidating the distribution of AChE in the brain and spinal cord of vertebrates. Histochemical analyses have shown that there are particularly high levels of AChE in the rat spinal cord and brainstem, (Koelle, 1954; Giacobini,1959; Navratnam & Lewis, 1970). There are also high levels in the cerebellum of rat although there is uncertainty as to its precise localization,(Silver,1967).

Much of the modern work is based upon studies involving investigation of nervous tissue grown in culture as these preparations have been shown to resemble in vivo preparations very closely, (Bunge, Bunge & Peterson,1965).

In tissue cultures of spinal cord, large cells have been observed staining for AChE and these have been suggested to be motoneurons (Hosli, Hosli & Wolf, 1975). In these brainstem cultures, more AChE staining neurons were observed in the areas of the hypoglossal nucle us and the nucleus ambiguus, while in cerebellar cultures AChE containing fibres resembling Purkinje cells were observed.

Recently, regional studies have been made of AChE in the brain of pig (Knutsen, Stanton & Shirachi, 1975) and in the central nervous system of the frog. (Nistri et al, 1975). Both groups showed definite regional differences. In the frog brain, the telencephalon showed the lowest concentration of enzyme and the rhomboencephalon the highest while the spinal cord had the highest levels of AChE in the cervical portion and lowest levels in the thoracic section. In the pig brain, the caudate nucleus showed the highest activity of AChE and the cerebral cortex the lowest. Cerebellar cortex always showed fairly high levels of enzyme although this is difficult to explain as ACh and choline acetylase levels were very low. (Silver, 1967)

2. Isolation

The present work has been complicated by the fact that AChE is bound fairly tightly to membranes and this factor has had to be considered during the extraction and purification steps. (Nachmansohn, 1971).

A. Extraction

Methods of solubilizing AChE from the membrane have depended largely on the source of the enzyme. The tissuesmost commonly used have been electric organ tissue (Nachmansohn & Lederer, 1939), erythrocyte stroma (Cohen & Warringa, 1953) and brain (Morton, 1950). The various techniques used reflect how different workers view the relationship of AChE with the membrane. Singer and Nicolson (1972) categorise membrane proteins as integral or peripheral. By their definition, AChE may be a peripheral protein because it can be extracted by the relatively mild procedure of high ionic strength media from erythrocyte ghosts (Mitchell & Hanahan 1966), and electric tissue

(Silman & Karlin, 1967), or the incubation of brain in ion-free media (Chan, Shirachi & Trevor, 1972; Hollunger & Niklasson, 1973), or increasing the pH of incubation of brain tissue (Hayden, Taylor, Forrest & Shirachi, 1973). However, Aloni & Livne (1974) point out that Singer & Nicolson's definition is equivocal because for example the AChE is only dissociable at high ionic strength from erythrocyte ghosts but not from the intact erythrocytes (Mitchell & Hanahan, 1966). Also various other workers have had to resort to using more severe techniques for removing the enzyme from the membrane, notably detergents. Paniker, Arnold, Hartmann (1973) studied the efficacy of several solubilizing agents such as detergents, chelating agents and salt, and they came to the conclusion that AChE is an integral protein of the erythrocyte membrane because removal of the enzyme was not selective but followed the solubilization of other membrane proteins. Jackson & Aprison (1966) and Ho & Ellman (1969) originally used detergents to solubilize the enzyme and they found an almost quantitative shift of activity from the particulate fractions into the 100,000g supernatants. Since then, the use of non-ionic detergents, particularly Triton X-100 to extract AChE has become almost routine due to their great efficiency. (see Wright & Plummer, 1972; Bellanger, Bouillon & Uriel 1973; McIntosh & Plummer, 1973; Leterrier, Rieger & Mariaud, 1974; Devonshire, 1975).

It has been found that many membrane enzymes tend to be denatured by certain detergents particularly those that are ionic such as sodium dodecyl sulphate and, to a lesser extent, the bile salts such as sodium deoxycholate (Coleman, 1973). The most successful ones have been the non-ionic detergents such as the Brij, Triton and Lubrol series'. Helenius & Simons (1975) suggest that this is due to their efficiency in dispersing the membrane lipid.

The electroplax (electric organ) of various electric fish is somewhat anomalous in that it has very high levels of AChE and acetylcholine receptor (AChR) and very little of any other protein. This fact has made the electric fish an obvious candidate as a source of the enzyme. The actual

electroplax has been shown to be phylogenetically derived from muscle (Nachmansohn, 1959). Extraction procedures have involved treating the electroplax with high ionic strength solution or low ionic strength media with trypsin but it would be fruitless to compare this organ with preparations of mammalian nervous tissue as it is so specialized in its action.

B. Purification

i. Older Methods. Early purification procedures could only be applied originally to the crude enzyme obtained from eel electric organ as this was the only source known to contain AChE in quantities large enough to obtain a reasonable yield of enzyme after all the purification steps.

Rothenberg & Nachmansohn (1947) introduced the use of ammonium sulphate fractional precipitation of the crude enzyme obtained from the Electrophorus electricus electroplax. This procedure was modified by Lawler (1959) who obtained a preparation with a recovery over the original crude enzyme of 15%. Kremzner & Wilson (1963) improved the technique by homogenizing the eletric organ in 5% ammonium sulphate and then sequentially chromatographing the enzyme through benzyldiethylaminoethyl cellulose, Sephadex G-200, Cellex-P and DEAE-cellulose. This protocol gave a yield of 9% with activity of 660 U/mg protein. This represented a purification of about 370. Leuzinger and Baker (1967) using the same technique have obtained crystals of the enzyme with an activity of 750 U/mg. protein and these are now being used in the determination of the crystal structure (Leuzinger, Baker & Cauvin, 1968; Chothia & Leuzinger, 1975).

Since the advent of affinity chromatography however, these older classical methods of preparing AChE have become to a great extent obsolete, and a detailed review is unnecessary.

ii. Affinity chromatography. The technique of affinity chromatography has, in the last decade, provided a major breakthrough in the purification of macromolecules. The great advantage is that the method is less time consuming than the classical purification procedures and the basic principle is fundamentally simple. The crude preparation of the macromolecule is

passed through a column which contains an insoluble matrix to which has been covalently bound a specific competitive ligand. Those contaminants which have no affinity for the ligand pass through unretarded while those which do possess an affinity are retarded to an extent depending on the degree of attraction to the ligand.

The origins of affinity chromatography may be found in the work of Kremzner & Wilson (1963). They introduced the concept of resin-bound catalytic site ligands by chromatographing AChE through benzylated DEAE-cellulose with the idea that the quaternary ammonium ligands would retard the enzyme. Froede & Wilson (1970) showed however that few quaternary groups formed and the column functioned as an ion exchange resin. Cuatrecasas and his co-workers first looked into the process of affinity chromatography as a viable technique when they attempted to purify the enzymes staphylococcal nuclease and α-chymotrypsin (Cuatrecasas, Wilchek & Anfinsen, 1968, Cuatrecasas & Anfinsen, 1971a and 1971b). The technique was then applied to AChE from electric eel electroplax and erythrocytes by Kalderon, Silman, Blumberg & Dudai, (1970) and Berman & Young,(1971).

As affinity chromatography has burgeoned over the last ten years, so it has become apparent that several factors affect the efficiency of the process. These involve the choice of spacer arm and ligand, concentration of attached ligand and the choice of matrix.

O'Carra has frequently drawn attention to the need to choose a suitable spacer arm separating the matrix from the ligand. He has stressed that any steric hindrance by the resin or spacer arm should result in a decrease in retardation of the molecule (O'Carra, Barry & Griffin, 1973). One could therefore be fairly certain that any retardation is biospecific. He has shown that in several systems adsorption of the macromolecule to the column has been due to non biospecific ion-exchange or hydrophobic interactions, (O'Carra, 1974a), and that the spacer arm acts as the principal adsorping moiety rather than the biospecific ligand. He suggests that this problem might be remedied to a certain extent by making the spacer arm more hydro-

philic. (O'Carra, Barry & Griffin, 1974a; O'Carra, Barry & Corcoran, 1974b). In fact he has shown that by hydroxylating the hydrocarbon spacer arm of an affinity column there was a substantial decrease in the non-biospecific adsorption of NAD linked dehydrogenases. (Barry & O'Carra, 1973). However, he says it might be desirable to carefully control the non-biospecific interactions rather than completely eliminate them on the grounds that many immobilized ligands furnish weak biospecific affinity. Thus if some non-biospecific interaction was allowed to reinforce the bioaffinity without dominating it, one could still obtain a useful affinity system. This mixed adsorption he terms 'compound affinity'. (Barry & O'Carra, 1973). The observations of O'Carra's group on non-biospecific adsorption of macromolecules to the spacer arm are substantiated by the work of several groups. Goodkin & Howard (1974) showed that the percentage of AChE adsorped to an affinity column increased as more methylene groups were introduced into the spacer arm. Similarly, Berman & Young (1971) increased the retention of AChE on an affinity column by doubling the N-succinyl-3,3′-diaminodi--propylamine spacer arm. However Goodkin & Howard (1974) also demonstrate the necessity of a spacer arm by showing that when the affinity ligand is bound directly to the column matrix, no AChE is retarded, and Blumberg & Katchalski (1970) have shown that ligand-enzyme interactions are disturbed when the ligand is close to the polymeric matrix.

The actual attachment of ligand to the polymeric backbone such as Sepharose can be done in two different ways. One procedure involves building up the affinity column sequentially by several steps of activation, spacer arm attachment and then ligand binding; the second procedure involves synthesis of the space arm-ligand as one molecule and then attaching it in one step to the activated Sepharose (Rosenberry, 1975). The first procedure allows unreacted materials and byproducts to be washed free from the gel in readiness for the next step. The second method however allows the concentration of ligand to be attached to the gel to be more finely controlled (Rosenberry, Chang & Chen, 1972). Also, this method ensures that

there are no unreacted spacer arms which would bind the protein non-biospecifically, (O'Carra et al, 1973)

The purification of AChE by affinity chromatography has been investigated by several groups during the last few years principally using electric eel electroplax as the source of enzyme. The high activity of AChE in this electric organ has allowed purification of the enzyme several thousandfold. Dudai, Silman, Kalderon & Blumberg (1972a) used [N-(ε-aminocaproyl)-p-aminophenyl] trimethylammonium bromide hydrobromide covalently linked to cyanogen bromide activated Sepharose in order to purify electric eel AChE. This column was only suitable really for purifying the non-aggregating 11S species of AChE. The reason for this was that the 14S and 18S species aggregated in low ionic strength media (Massoulié & Rieger, 1969; Dudai et al, 1972a), yet it was necessary to maintain low ionic strength during elution because it has been shown that the inhibition constants of AChE inhibitors decrease with increasing ionic strength (Changeux, 1966). Thus if high ionic strength media had been used, the immobilized ligand would not have functioned as an affinity column. (See later in this thesis for an explanation of the AChE multiple molecular forms).

The above drawbacks led Dudai, Silman, Shinitsky & Blumberg (1972b), to develop an AChE inhibitor which, when covalently linked to Sepharose, would form an affinity column that could retard the enzyme even at high ionic strength. The ligand-spacer arm conjugate they developed was 1-methyl-9-[N$^{\beta}$-(ε-aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide (MAC). Their results showed that 89% of the AChE applied was retarded on the column in 1 mol/l NaCl and about 50% of the applied enzyme could be eluted by passing the competitive AChE inhibitor decamethonium bromide through the column. The purified enzyme was shown to have both the 14S and 18S sedimenting forms present and both of these aggregated at low ionic strength.

Several other groups have used the phenyltrimethylammonium (PTA) ligand when attached to various spacer arms as the AChE retarding inhibitor.

Berman & Young (1971), purified the eel AChE using the meta and para derivatives of this ligand but only the meta derivative was successful in retarding the erythrocyte enzyme.

Similarly, Chan, Shirachi, Bhargava, Gardner & Trevor (1972), working with bovine brain, Yamamura, Reichard, Gardner, Morrisett & Broomfield (1973) working with guinea pig brain, and Dawson & Crone (1974) working with bovine brain showed the greater retention of AChE on columns containing the meta-PTA ligand rather than the para-PTA ligand.

Dawson & Crone (1974) also compared elutions when Triton X-100 was present with elutions when the detergent was absent. This is of particular interest when purifying Triton solubilized AChE as will be seen in this thesis. They showed that an additional peak of activity was eluted when the detergent was incorporated into the elution media. This agrees with Crone's (1971) data in which he found that some Triton solubilized enzyme adsorped to an agarose column if thedetergent was not present during elution. However, it is not consistent with the work of Yamamura, Reichard, Gardner, Morrisett & Broomfield (1973) who managed to purify Triton solubilized guinea pig brain AChE without including Triton in the elution buffer. Dawson & Crone (1974) explain this by saying that the detergent present with the applied enzyme maintained high enough levels of detergent throughout all stages of the procedure. This conclusion does not agree with findings in this thesis (see Results and Discussion). Ott, Jenny & Brodbeck, (1975), used affinity chromatography to purify Triton solubilized erythrocyte AChE but the detergent was not present in the elution media. However, they do stress that Sephadex gel seive chromatography of the enzyme gave different results when Triton X-100 was excluded from the system as opposed to when the detergent was present. Goodkin & Howard (1974) in their purification of rat brain AChE by affinity chromatography mention that when Triton X-100 was omitted from the gel, the enzyme recovery was markedly reduced. Grobmann & Liefländer, (1975) however, purified detergent free erythrocyte AChE by 158,000 fold with a 28% yield by 2 steps of affinity

chromatography. It is possible that the differences mentioned above by different workers reflect the different ligands used during the procedures or the varying sources used for the AChE.

3. Structure

The IUPAC-IUB (1971) defined the term 'multiple forms of the enzyme' as a broad term covering proteins possessing the same enzyme activity and occurring naturally in a single species. This description applies to AChE rather than the term isoenzyme which, strictly speaking, applies to multiple forms of the enzyme arising from genetically determined differences in primary structure, and not to those derived by modification of the same primary sequence.

Efforts at determining the molecular weight and sub-unit composition of AChE have mainly been directed at the enzyme present in electric organ. Studies on partly or highly purified preparations using polyacrylamide gel electrophoresis or density gradient centrifugation have shown that AChE exists in several multiple molecular forms of varying molecular weights.

A. Molecular weight and sub-unit composition

Rothenberg & Nachmansohn (1947) were the first workers to suggest that AChE existed in several forms from the nature of the sedimentation coefficients. Lawler (1963) indicated the presence of different AChE molecules ranging in molecular weight from $3{\cdot}3 \times 10^5$ to $3{\cdot}1 \times 10^7$ and consisting of multiples of the same sub-unit. She also stressed that a polymer with molecular weight of about 30 million could not be present in the membrane as a sphere but might exist as a flexible rod.

Since these early findings several laboratories have shown conclusively that AChE in solution exists in different multiple molecular forms (Hargreaves, Wanderley, Hargreaves & Gonzalves, 1963; Massoulié & Rieger, 1969; Dudai et al, 1972a). There seems to be fair agreement, especially between Massoulié's group and Dudai's group as to the sub-unit composition of the enzyme from electric organ. The predominating species (by sedimentation studies) are the 9S, 14S and 18S forms which aggregate

at low ionic strength, and the 11S and 8S species which do not show this aggregating phenomenon (Massoulié, Rieger & Bon, 1971; Rieger, Tsuji & Massoulié, 1972; Rieger, Bon & Massoulié, 1973a). Similar findings have been obtained by Grafius & Millar (1965), Dudai et al, (1972a), Dudai, Herzberg & Silman (1973). Electron microscopic work by Dudai et al (1973), and Rieger et al (1973b), have shown that aggregating species of 9S, 14S and 18S are "grape like" in that they consist of several sub-units from which projects a long tail. The non-aggregating species on the other hand do not have this tail and are thus more globular. Although there is as yet no proof, Dudai says that it is tempting to speculate that the tail structure is involved with fixing the enzyme to the membrane. In addition to the aforementioned AChE species, a "dumbbell" shaped molecule has also been observed which seems to be composed of two clusters of AChE sub-units joined by a "tail like" structure,(Dudai et al, 1973; Wermuth & Brodbeck, 1972).

The various forms of the enzyme reflect to a large extent the methods of solubilization from the membrane or how the solubilized forms are treated. Proteolysis solubilization or trypsin treatment yielded the globular non-aggregating forms (Massoulié et al, 1972a; Massoulié, Rieger & Tsuji, 1970) whereas solubilization by high ionic strength yields the aggregating species resembling the 'bunch of grapes' under the electron microscope. (Massoulié et al, 1971). Wermuth, Ott, Gentinetta & Brodbeck (1975) have summarised the interconversions diagramatically (see Fig.I.1)

The sub-unit molecular weight has caused much argument and the actual value has still not been conclusively resolved. Leuzinger, Goldberg & Cauvin,(1968) after centrifuging AChE in guanidine and subjecting it to SDS gel electrophoresis, concluded that the enzyme was a tetramer (MW 240,000) consisting of two different sub-units of molecular weight 64,000, thus giving a dimeric hybrid $(\alpha\beta)2$. However, Dudai & Silman (1972) found two species of sub-unit; one in the molecular weight range of 82,000-100,000 and the other about 59,000, the tetramer having a MW of 320,000-350,000. This is verified by Massoulié's

laboratory (which favours the dimeric hybrid) where similar results were obtained by Powell, Bon, Rieger, & Massoulié, (1973) who referred to the sub-units as light (l) and heavy (h) and who denote the tail as 'q'. Using this explanation it is possible to obtain a whole range of molecular weights by using different permutations of the different sub-units. Wermuth et al (1975) have summarised the different combinations of sub-units (see Table I.2). Dudai & Silman (1974) proposed that the 59,000 fragment was an autolysis product of the larger sub-unit and a polypeptide fragment of MW 25,000 was lost during the process. They do not discount the Leuzinger et al, results, but instead suggest that they have in fact been analysing the proteolysed enzyme and also that it might be possible for the tetramer to be proteolysed and yet still maintain its tetrameric structure (see Fig.I.2).

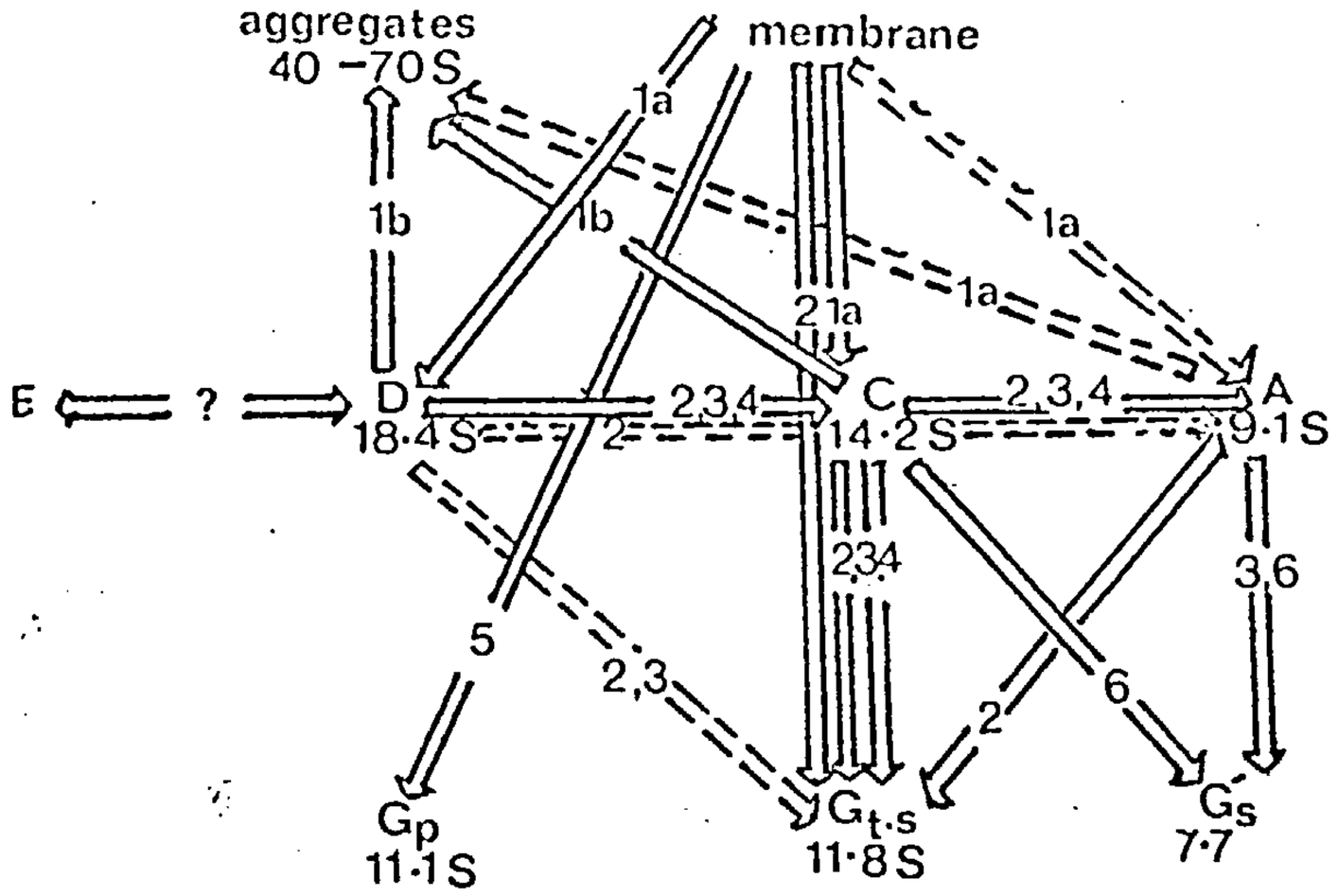
Rosenberry, Chen & Bock (1974) disagree with the dimeric hybrid $(\alpha\beta)2$ model and propose that the tetramer is composed of identical sub-units as a dimer of dimers $(\alpha 2)2$. In their results they show that the α sub-unit may be cleaved into 2 polypeptides, one of which may be further cleaved into 2 more polypeptides. They suggest that the dimeric hybrid $(\alpha\beta)_2$ model has been based on observations of the sub-units at various stages of proteolytic cleavage.

Recently Cartaud, Rieger, Bon & Massoulié (1975) have published results indicating that the higher MW polymeric forms of AChE are composed of an assembly of tetrameric units. From electron microscopic evidence they suggest that form D (see Table Fig.I.2) is composed of 3 tetramers. These are joined by 3 filaments which project from the tetramers and come together eventually to form the tail in an α-helical secondary structure.

Some studies have also been performed on AChE isoenzymes from other sources such as erythrocytes, brain and diaphragm although results have not proved to be so fruitful as the electric tissue enzyme. Hollunger & Niklasson (1973) isolated AChE from bovine brain with a MW of 80,000 which aggregated on storage. If the enzyme was prepared in the presence of

Fig.I.1

Ways of Solubilization of AChE from the membrane and the interconversions among the various forms.



1a, High ionic strength; 1b, low ionic strength; Ca^{++}; 2, trypsin; 3, sonication; 4, spontaneous; 5, autolysis; lipase; 6, Triton X-100

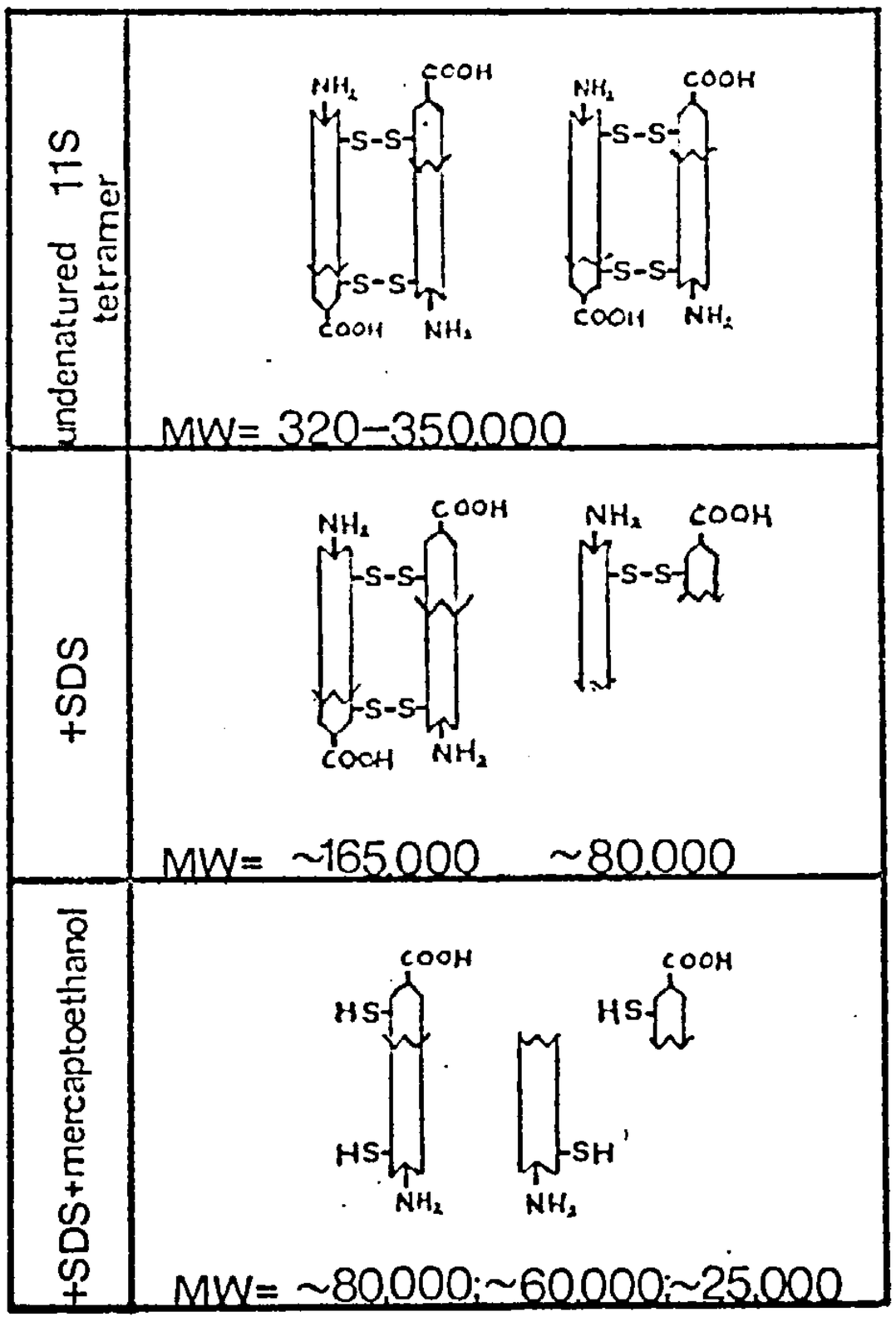
Table I.2

Properties of Elongated and Globular Forms of AChE

Form	Subunit composition	Molecular weight (daltons)	Specific activity (U/mg)	Stoke's radius (nm)	Sediment. coefficient (S)	Occurrence in EM.
Elongated						
A	h_2l_2q	430,000		12·4	8·5–9·1	grape-like
C	h_4l_4q	780,000	14,300	14·4	14·2	grape-like
D	h_6l_6q	1,100,000		15·0	18·4	grape-like
E	h_8l_8q	1,560,000	13,300	17·0	–	dumbbell
Globular						
G_p	h_2l_2	260,000	15,200	8·2	11·1	tetramer
G_t	h_2l_2	290,000	–	–	11·8	tetramer
G_s'	hl	155,000	–	6·4	7·7	dimer

Fig.I.2

Schematic model of subunit structure of 11S AChE together with the products to be expected under the conditions employed for SDS - polyacrylamide gel electrophoresis, both in the presence and absence of β mercaptoethanol subsequent to cleavage of either one or both of the polypeptides of the dimer. The site where cleavage can occur is indicated by a serrated line.



DEAE-Sephadex-A25, the enzyme no longer aggregated suggesting the removal of an aggregating factor. Viana, Chan & Trevor (1974) using the same source isolated three MW species of 120,000, 220,000 and >500,000. They found that the smaller form of the enzyme aggregated eventually through the intermediate form to the large MW molecule. McIntosh & Plummer (1973) detected between two and six forms of pig brain AChE, the most frequently found being 60,000, 130,000, 198,000, 266,000 and 350,000. Results in this thesis show an extension of the work of McIntosh & Plummer (1973).

Ott et al (1975) obtained a MW value of 80,000 for the AChEsub-unit from the detergent solubilized human erythrocyte. They showed that the enzyme in the presence of detergent exists in various forms differing in net charge but of similar molecular dimensions. They also show however, that removal of Triton X-100 causes the formation of AChE of different molecular sizes. On the other hand, Wright & Plummer (1973) demonstrated that Triton X-100/KCl solubilization of erythrocyte enzyme resulted in several MW forms of AChE even in the presence of Triton X-100.

The above review of results illustrates the varying findings published by different laboratories. However, there does seem to be some correlation in the results suggesting a basic MW species of approximately 60,000 and/or 85,000.

Whether the enzyme exists in the membrane as a monomer or a multiple molecular aggregate has not been definitely elucidated. With the demonstration of the existence of a protein tail projecting from a bunch of several sub-units, it would be attractive to suggest a multiple molecular aggregate being rooted on the membrane via the tail. However, recently Levinson & Ellory (1974) have shown that the form of enzyme in the membrane is a monomer of molecular weight 75,000 and that multiple forms of the enzyme observed in solubilized preparations are probably aggregates of this monomer.

The actual composition of the enzyme has been reported to be of a glycoprotein nature (Powell, Bon, Rieger & Massoulié, 1973) giving a positive sialic acid assay and staining for carbohydrates. It is suggested this may

be significant since many surface membrane proteins are glycoproteins and that the glycoprotein nature of AChE would agree with its suggested location at the synaptic membrane surface. It has also been put forward that phospholipid is involved with AChE because phospholipase C converts the aggregating forms of the enzyme into non-aggregating forms. (Rieger, Bon & Massoulié, 1973b). This conclusion is also supported by Sihotang (1976) for erythrocyte AChE who also showed that preparations of the lipid free enzyme were activated approximately 5-fold on the addition of the phospholipid phosphatidyl serine.

B. Equivalent weight

The equivalent weight of a protein can be defined as its grams per mole of active sites. It is possible to determine this value provided that a suitable titrating agent can be found specific for the active site and the protein is very pure.

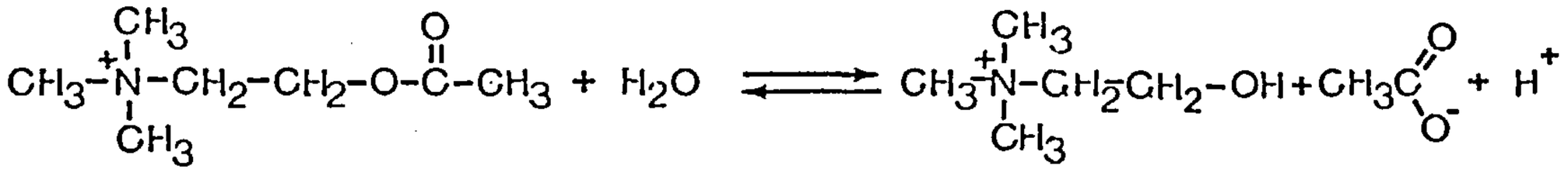
Many AChE titrants used have functioned by acylating the serine in the active site. Michel & Krop (1951) used radioactive DF^{32}P to determine the normality of the enzyme, whereas Kremzner & Wilson (1964) followed the loss in activity concommitant with phosphorylation by N,N-Dimethyl-S-(diethyl--phosphoryl) thioethanolamine. Another technique is to bind fluorescent ligands which exhibit diminished quantum yields when associated with the enzyme (Rosenberry & Bernhard 1971; Mooser, Schulman & Sigman 1972;) or which are totally quenched when bound (Mooser et al, 1972;). More recently Taylor & Lappi (1975), used the ligand propidium which binds to a locus peripheral to the catalytic site and shows a ten -fold increase in fluorescence when bound.

Rosenberry (1975b) arrived at a value for the equivalent weight of AChE of 76,000g/mole of active sites which is close to the sub-unit molecular weight of eel 11S enzyme. The number of active sites per tetrameric molecule is thus the MW divided by equivalent weight which is the number of sub-units per molecule. The value as determined by several workers is four active sites per tetrameric molecule (Mooser et al, 1972; Chen, Rosenberry & Chang (1974).

4. Catalysis

A. Catalytic mechanism

AChE is a serine hydrolase along with other esterases and peptidases and show irreversible active site phosphorylation, (Cohen & Oosterbaan, 1963; Rosenberry, 1975a). The reaction takes place as follows:



The peptide chain in the region of this residue has been shown to be very similar to the active site of other enzymes within this classification such as chymotrypsin and elastase (Schaffer, Michel & Bridges, 1973). The structure of the active centre of AChE is very complimentary to acetylcholine. The substrate has a quaternary nitrogen which is consequently positively charged and also an ester bond. The enzyme active centre correspondingly possesses a negatively charged 'anionic site' which attracts the quaternary nitrogen and orientates the ester bond over an 'esteratic site' which is responsible for the hydrolytic process. (Wilson & Bergmann 1950a; Krupka, 1964).

The hydrolytic process takes place in two stages. The enzyme combines with the substrate to form the enzyme-substrate (Michaelis-Menten) complex which is then hydrolysed to the final products. The catalytic mechanism of chymotrypsin has often been used as a model to explain that of AChE. Chymotrypsin has been shown to have a characteristic "charge-relay" system of hydrogen bonds comprising the active site serine hydroxyl, the imidazole side chain of histidine and the carboxyl group of aspartate (Blow, Birktoft & Hartley, 1969). The acylation or deacylation by substrate of the enzyme shows general base catalysis by the imidazole (Bender, Clement, Kézdy, D'A Heck, 1964) and it is suggested that in the charge relay system the imidazole withdraws protons from the serine hydroxyl as the substrate acylates the enzyme. The analogy is drawn with AChE because it also shows an apparent pKa of between 6-7 which indicates general acid-base catalysis

involving an imidazole group. Experiments on the pH dependance of hydrolysis by AChE have shown that the enzyme relies on groups which ionize at pH 6·5 and 9·4, (Krupka & Laidler, 1960). These values are similar to those(pH 6·2 and 10·1) which are found during the reaction with saturating acetylcholine. The characteristic bell shaped curve of activity versus pH showing maximal activity between pH 8-9 indicates the dissociation of acidic and basic groups. Krupka (1967) has shown that the activity of AChE also depends on a second imidazole the basic group of which has a pKa 5·6. He suggests that this imidazole acts as a general base in acetylation and the other imidazole (pKa 6·5) has the role of a general base in de acetylation and also stops cation binding at the active site when protonated. This mechanism is not generally favoured. (Rosenberry, 1975b). Also, the view that the basic group with a pKa of 6·5 is the enzyme nucleophile has been discarded in favour of the serine hydroxyl, (Froede & Wilson 1970).

A more likely mechanism is Brestkin & Rozengart's (1965) explanation of the model of Wilson, Bergmann & Nachmansohn (1950) (see Fig.I.3). The single imidazole group in the active site increases the nucleophilicity of the serine oxygen. A covalent bond is then formed between the oxygen and the carboxyl group in acetylcholine and the choline is then released. Deacetylation follows the reverse process as the acylated enzyme is hydrolysed.

Froede & Wilson (1970) explain the mechanism as follows:

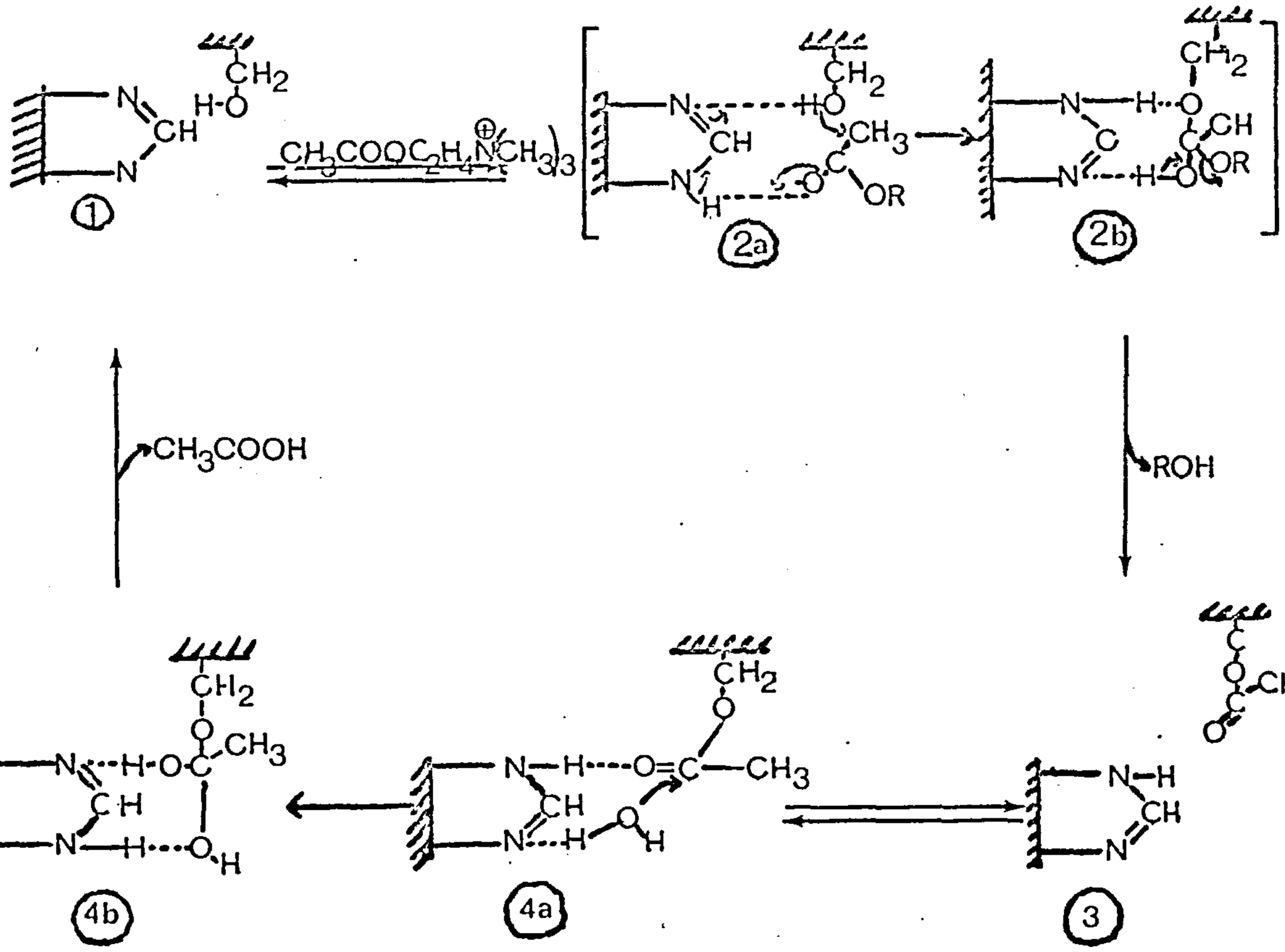
$$E + S \rightleftharpoons E{\cdot}S \rightleftharpoons E'{\cdot}P_1 \rightleftharpoons E' + P_2 \xrightleftharpoons{H_2O} E{\cdot}P_2 \rightleftharpoons E + P_2$$

where S is substrate, E.S is the Michaelis complex between enzyme and substrate, E'.P is the Michaelis complex between acetyl enzyme and choline, E' is the acetyl enzyme, $E{\cdot}P_2$ is the Michaelis complex between acetic acid and enzyme, P_1 is choline, P_2 is acetic acid. When P_1 and P_2 are in concentrations low enough not to affect the kinetics, the dissociation of complexes is rapid.

$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E{\cdot}S \longrightarrow E' + P_1 \xrightarrow{H_2O} E + P_2$$

Fig. I.3

AChE Hydrolysis of ACh
(Brestkin & Rozengart 1965)



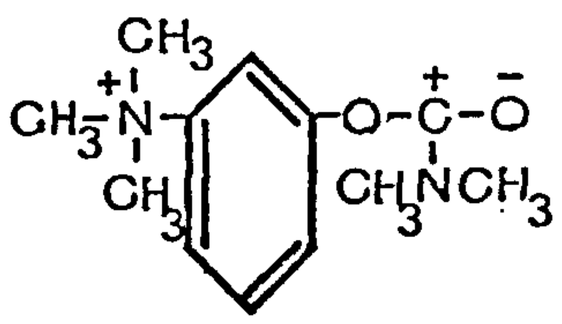
The Michaelis-Menten steady state equation thus give the reaction velocity

$$v = \frac{V_{max}}{\frac{Km}{S} + 1}$$

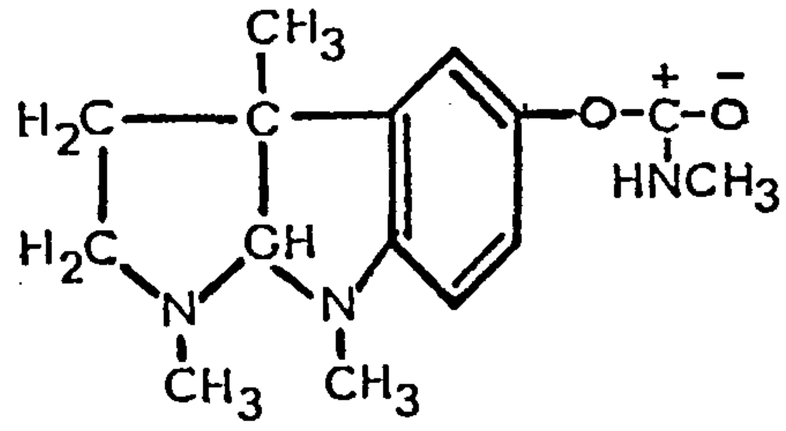
B. Inhibition

Much information has been gained about the active site structure of AChE with the use of active site inhibitors (both anionic and esteratic site inhibitors).

i) Anionic site. Wilson & Bergmann (1950a) first demonstrated the presence of a negative centre in AChE with the aid of competitive inhibitors. Two competitive inhibitors of similar structure which they used were physostigmine and prostigmine. However, these two compounds differ in that prostigmine is a quaternary ammonium ion, and consequently is positively charged at all pH's; physostigmine is a tertiary amine and changes from conjugate acid at pH6 to a neutral molecule at pH 10. Therefore physostigmine begins to lose its effectiveness as a ligand for the anionic site above pH6 whereas prostigmine does not.



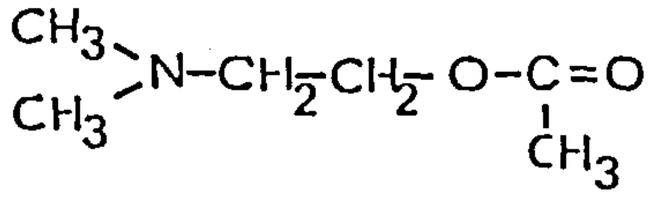
Prostigmine



Physostigmine

Hestrin, (1949) and Wilson & Bergmann (1950b) showed a similar effect with substrates. Dimethylaminoethyl acetate is a conjugate acid below pH 8·3 and is consequently cationic whereas at higher pH's it is uncharged. Likewise it is hydrolysed rapidly by AChE at pH's between pH 8-9 but above this range it loses its effectiveness as a substrate. On this basis of the electric charge upon substrates and its effect on enzyme binding, the mode of binding of acetylcholine to AChE has been accepted as ionic bonding to

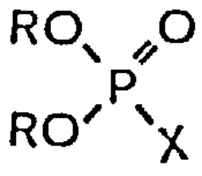
an anionic site.

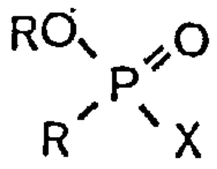


Dimethylaminoethyl acetate

However, Wilson (1952) showed that in addition to the above mentioned coulombic forces, unspecific van der Waal's forces contributed to the binding of molecules to the anionic site. Using the competitive inhibitor hydroxyethylammonium at pH 7 (cationic) he observed the effect on binding to the anionic site of sequentially methylating the nitrogen. He found that each alkyl group increased binding seven fold except for the fourth methylation. He reasonably assumed this enhancement was due to van der Waal's attraction by the methyl groups to hydrocarbon moieties in the enzyme. The fourth methyl group would have little effect due to the tetrahedral nature of the molecule stopping the group from being close to the enzyme.

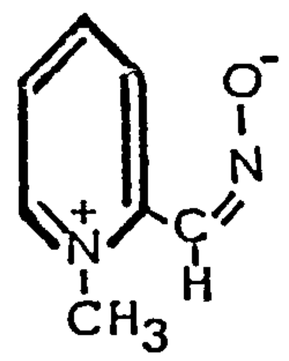
ii) Esteratic site. The process of catalysis at the esteratic site has already been discussed under the heading of 'Catalysis', and the point been made that the carbonyl group of acetylcholine binds covalently to the serine oxygen. Much understanding of the esteratic site has been gained by the use of organophosphate inhibitors, and has led eventually to the development of nerve gases, insecticides and consequently to antidotes. The mechanism of organophosphate inhibition is that the electrophilic phosphorus atom forms a covalent bond with nucleophilic group in the esteratic site with the elimination of an acidic group such as F^- (Wilson & Bergmann, 1950a). However, unlike the acetylated enzyme, the phosphorylated AChE is hydrolysed extremely slowly or not at all by water. Organophosphates have the general formula:





where $X = F^-$, Cl^-, CN^-, O_2N-C$_6$H$_4$-O^-. i.e. X = an acidic group.

Organophosphates could be regarded as substrates as they bind to the esteratic site optimally at pH 8 which is the same as substrates (Wilson & Bergmann, 1950a). Wilson (1951) showed that hydroxylamine (a nucleophilic reagent) reactivated organophosphate inhibited AChE by 50% within 5 hours by competing for the electrophilic phosphorus atom in the esteratic site. He then suggested that if such a nucleophilic reagent could be linked to a cationic nitrogen which would bind to the anionic site of AChE, the reactivating power would be enormous. This led to the development of 2-pyridine aldoxime methiodide (2-PAM) which was found to reactivate AChE which had already been inhibited by DFP. (Wilson & Ginsburg, 1955)

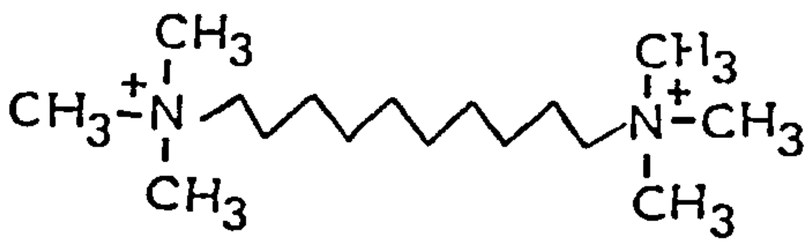


2- PAM

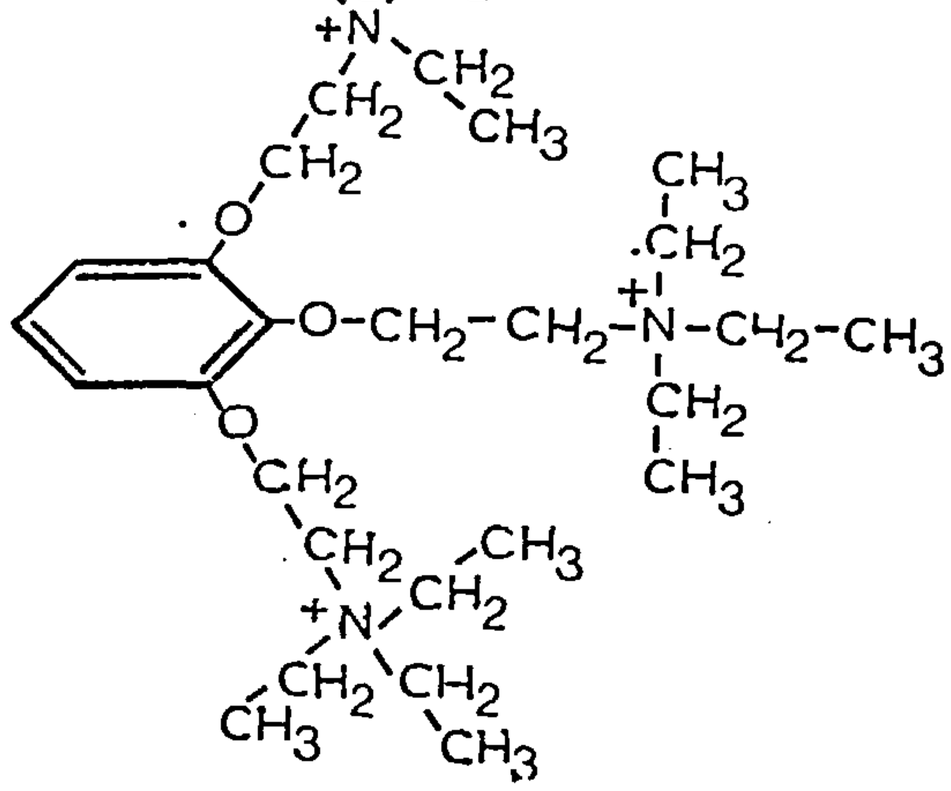
C. Peripheral anionic sites & Induced fit.

Evidence of a peripheral anionic site (P_1) is shown particularly with the bis quaternary inhibitor decamethonium bromide (Roufogalis & Quist, 1972). This compound inhibits AChE by spanning the peripheral anionic site and the catalytic anionic site. In addition, it has been demonstrated that flaxedil (which has three quaternary nitrogens) binds tightly to AChE

but the binding is not antagonized by active site inhibitors (Changeux, J-P, 1966) or by monoquaternary ligands which bind to the P_1 site (Roufogalis & Quist, 1972). Therefore the possibility of three more peripheral anionic sites (P_2, P_3, P_4) cannot be dismissed.



Decamethonium



Flaxedil

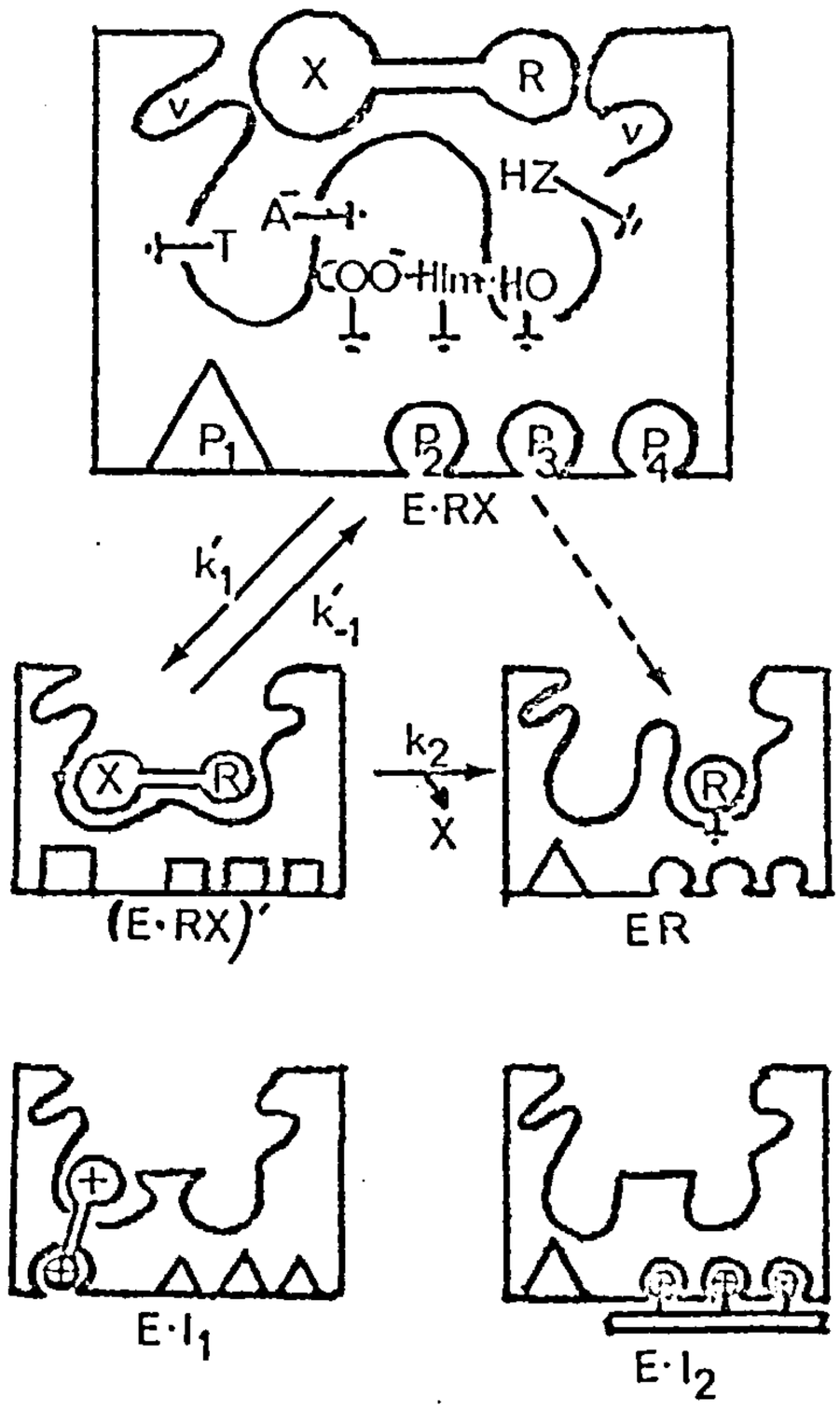
On the basis of these observations on the peripheral anionic sites, Rosenberry (1975b) has formulated an "induced fit model" for AChE (Fig.I.4) based on the induced fit model of Koshland (1958). The enzyme substrate complex E·RX is assumed to be in equilibrium with free E and RX and conformations of the catalytic site are similar for the free enzyme and in E·RX. The E·RX complex searches for the induced fit complex E'·RX' conformation which increases acylation by lowering the transition state for the covalent bond rearrangement in k_3. He suggests that reversible ligands which bind to peripheral sites modulate activity by reducing conformational flexibility and stabilizing catalytic site conformation; this reduces the transition state for acylation by poor substrates but varies it for good substrates. It is still too early to judge the general opinion of this model, but it does serve to explain the function of the anionic sites.

5. Biological Function

The biological significance of AChE becomes apparent when one notes that anticholinesterases severely disrupt electrical activity, (Bullock,

Fig. I. 4

Induced Fit Mechanism for AChE
(Rosenberry, 1975b)



The initial enzyme substrate is complex E·RX; the induced-fit complex, (E RX)′; and the acyl enzyme, ER. The enzyme-ligand complex with bisquaternary ligands like decamethonium ($E \cdot I_1$) involves the anionic site and peripheral site P_i; the complexes formed by other multi-quaternary ligands like flaxedil ($E \cdot I_2$) involve ligand binding at other peripheral sites (P_2, P_3, P_4). Identified residues at or near the catalytic site include the charge-relay complex (COO^- --HIm--HO; an acidic group HZ; the anionic group A^- which defines the anionic site; a tryptophan residue T near the anionic site; and adjacent hydrophobic areas V.

Nachmansohn & Rothenberg, 1946). These effects are most commonly seen in areas of excitable membrane which are not protected by structural barriers (myelin) such as autonomic ganglia and neuromuscular junctions. The importance of AChE is reflected in the efforts being put into the insecticide industry in developing anticholinesterases with species specificity. The role of AChE in the erythrocyte membrane is still as yet undetermined although it has been variously reported that it might function as a cation ionophore particularly for potassium (Giberman, Silman & Edery, 1973).

A. Acetylcholinesterase and the Acetylcholine Receptor

The fact that AChE and the acetylcholine receptor (AChR) coexist in excitable membranes indicates a very close relationship between the two glycoproteins and several authors still maintain that it has not been definitely disproved that they are one and the same molecule (Heilbronn, 1975). However, Simantov & Sachs (1973) have shown that there are large immunological differences between AChE and AChR which indicate they are in fact separate entities. The number of receptors and AChE active sites have been shown to be equal in both membrane preparations (O'Brien, Gilmor & Eldefrawi, 1970) and solubilized fractions (Changeux, Kasai & Lee, 1970). This stoichiometry is one of the reasons that led Neumann, Nachmansohn & Katchalski, (1973) to formulate the 'basic excitation unit' (see section I.5.Bi) which lends support to Nachmansohn's views on the chemical basis of nerve activity.

B. Acetylcholinesterase and excitability.

i) Integral model. Over the years a cyclic process has been formulated in which acetylcholine is released from a storage protein (S), binds on to the receptor (AChR) which causes the ion fluxes constituting nervous excitation, and is finally destroyed by the enzyme AChE. Nachmansohn has always maintained that this chemical process is fundamental to nervous excitability at excitable membranes in cholinergic neurones whether at the synapse or in the axonal membrane (Nachmansohn & Neumann, 1975). This theory is

questioned by Hodgkin's Cambridge group who cannot equate the chemical theory with the fact that 10^5 impulses per hour pass in the perfused squid axon even though most of the axoplasm has been removed. (Baker, Hodgkin & Shaw, 1962; Keynes & Aubert, 1964). However, both groups agree that chemical events are important at the nerve ending.

The various proteins of the Acetylcholine cycle (Fig.I.5) have now been rationalized into an 'integral model' by Neumann et al (1973) in which the storage, receptor and enzyme proteins are combined into a basic excitation unit (BEU) (Fig.I.6) which surrounds an ion gateway. The fundamental novelty of this system is that acetylcholine is processed continuously through the BEU's within the excitable membrane. This intramembrane concept contrasts with the widely held intercellular neurotransmitter theory (see Section I.5.Bii). In the integral model the generation of nerve impulses is brought about by a co-operative increase in the rate of ACh translocation through the BEU's. The model predicts that AChE is essential for the maintenance of the action potential in the membrane. The integral model brings together several facts and hypotheses in a controversial but attractive scheme which has already been questioned (Rosenberry, 1975). For a comprehensive review of the theory see Nachmansohn & Neumann(1975).

ii. Classical neurohumoral theory. In the generally accepted neurohumoral theory, a presynaptic nerve impulse causes the release of ACh through the presynaptic membrane into the synaptic gap and then combines with the receptors at the synaptic membrane. This combination brings about a conformational change in the membrane which causes an increase in permeability to the cations Na^+, K^+, Ca^+ and Cl^-. ACh is then removed by AChE.

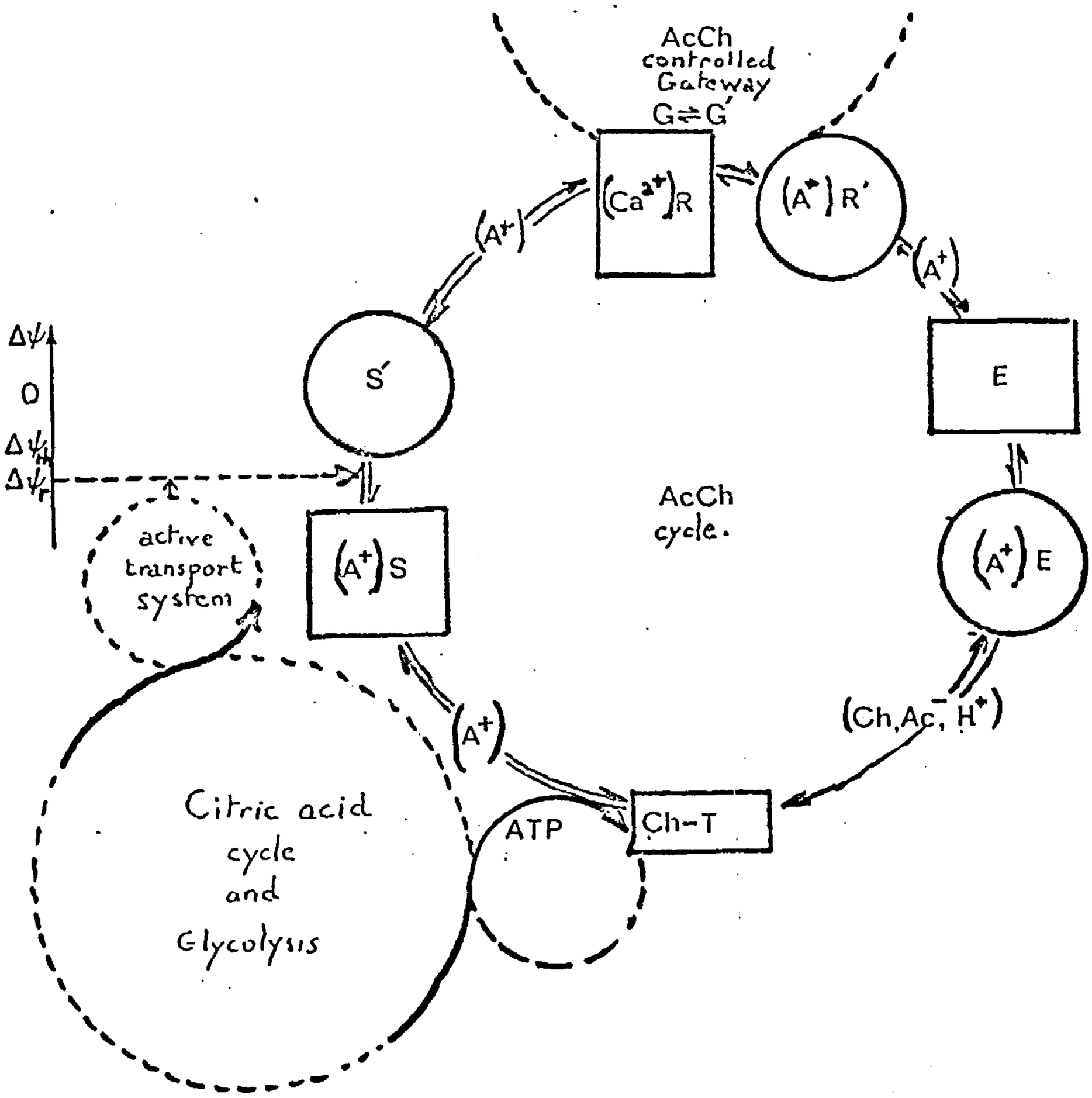
As the nerve axonal interior is rich in K^+ ions and the exterior is rich in Na^+ and Cl^- ions a negative potential of approximately -70mv exists in the cell interior. As the membrane is semipermeable, the ionic status quo is maintained by the Na^+. K^+ - ATPase (Katz,1966).

During a nerve impulse, the membrane potential is lowered below a threshold level whereupon there is a rapid, specific but transitory increase in

Fig. I.5

Acetylcholine Cycle

Nachmansohn & Neumann, 1975

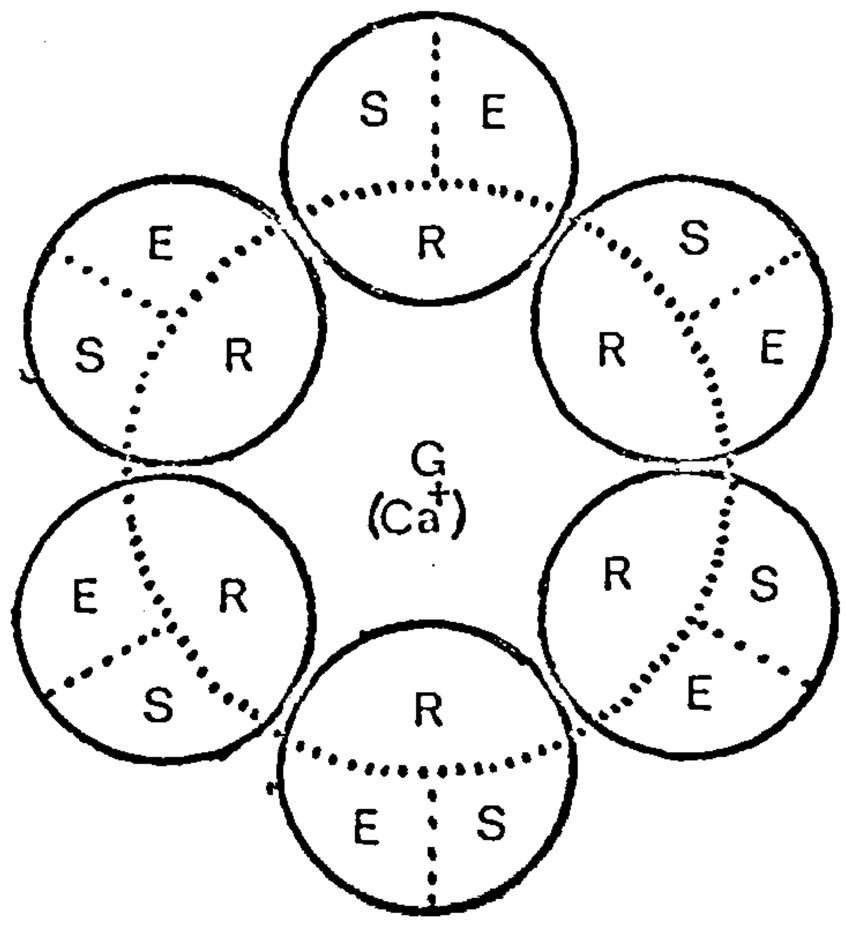


The binding capacity of the storage site for ACh is assumed to be dependant on the membrane potential $\Delta\psi$ and is thereby coupled to the active transport system and the citric acid cycle and glycolysis. The control cycle for the gateway G (Ca^{2+} binding and closed) and the G' (open) comprises the SRE assemblies (see Fig.I.6) and the choline O-acetyl transferase (Ch-T); Ch-T couples the ACh synthesis cycle to the translocation pathway of ACh through the SRE assemblies. The continuous subthreshold flux of ACh through such a subunit is maintained by the virtually irreversible hydrolysis of ACh to Choline (Ch^+), acetate (Ac^-), and protons (H^+) and by steady supply flux of ACh to the storage form by the synthesis cycle. In the resting stationary state, the membrane potential ($\Delta\psi r$) reflects dynamic balance between active transport (and ACh synthesis) and the flux of ACh (through the control cycles surrounding the gateway) and of the various ions unsymmetrically distributed across the membrane. Fluctuations in membrane potential (and exchange currents) are presumably amplified by fluctuations in the local ACh concentrations maintained at a stationary level during the continuous translocation of ACh through the cycle.

Fig.I.6

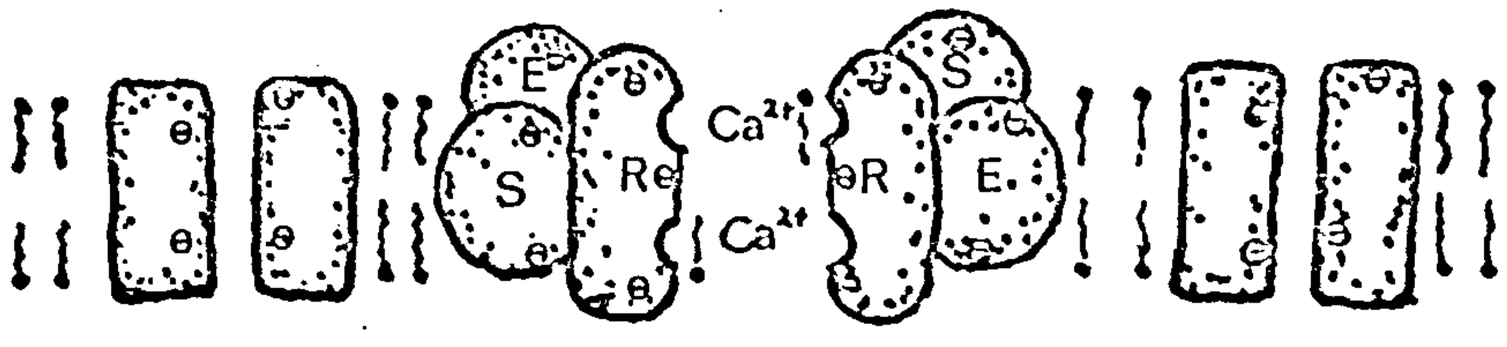
Schemes of AChE Controlled Gateway

(a)



(a) basic excitation unit (BEU) containing in this example 6 storage/receptor/enzyme (SRE) assemblies viewed perpendicular to the membrane surface.

(b)



(b) Cross section through a BEU flanked by 2 units with model ion passages for K^+ ions in the resting stationary state. The minus signs $^{\ominus}$ symbolize negatively charged groups of membrane components.
(after Neumann. see Nachmansohn & Neumann 1975)

sodium permeability (Hodgkin, 1951). The inward movement of Na^+ makes the inside of the nerve positive and the current carried by Na^+ spikes very quickly and falls almost as rapidly. During the descending phase of the Na^+ current, K^+ moves out in a much slower and less intense manner than Na^+. The ion channels appear to be independant as Na^+ conductance can be selectively blocked by tetrodotoxin and K^+ by tetraethylpyrophosphate.

At the nerve ending the increase in ion permeability is caused by the action of ACh at the postsynaptic membrane. The permeability changes at the nerve ending in contrast to the axon are thought to be unspecific (Fatt & Katz, 1952).

In the presynaptic nerve terminal, ACh is present in the soluble fraction and also in an occluded vesicular fraction. The demonstration by Whittaker Michaelson & Kirkland (1964) that the vesicles contained ACh lent support to the quantal theory of ACh release at the presynaptic membrane (Del Castillo & Katz, 1954) whereby the vesicles fused with the presynaptic membrane releasing the ACh into the synaptic cleft. Heuser & Reese (1973) have indeed shown electron micrographs of vesicles pinocytosing with the membrane. However, when AChE is injected into the axon which destroys the soluble ACh but not the occluded vesicular ACh, transmission is abolished. (Tauc, Hoffman, Tsuji, Hinzen & Faille, 1974). This evidence favours ACh release from the soluble fraction. Further investigation is needed to elucidate the correct mechanism of ACh release. For a critical review of the origin of synaptic ACh, see Marchbanks (1974).

After transmitter release the ACh diffuses to the receptor molecule which is an integral protein embedded in the lipoprotein matrix of the postsynaptic membrane. De Robertis (1971) has proposed that ions move through the membrane in response to an ACh induced conformational change in the AChR. Changeux & Podleski (1970) have adapted the 2-state model of Monod, Wyman, Changeux (1965) for allosteric interactions to the AChR. The receptor is proposed to exist in two conformations: a relaxed state R, and an active state S which corresponds to the depolarized membrane. These two states

are in equilibrium. The S state which causes ion permeability is stabilized by agonists (receptor activators) such as ACh, decamethonium and suxemethonium while the R state has preference for the antagonists (receptor inhibitors) such as curare and tetracaine.

Following membrane depolarization, the transmitter is rapidly hydrolysed by the enzyme AChE. This mechanism of transmitter removal differs from the adrenergic nervous system where the transmitter is actively reabsorbed into the presynapse. Wilson (1971) has shown that the concentration of AChE at the electroplax junction is 6×10^3 molecules per μ^3 which is higher than the ACh concentration. The turnover time of the enzyme is 30-40 μ secs, and since some nerve fibres conduct 1000 impulses per second it can be seen that AChE is efficient enough to remove the transmitter after the spike has reached its peak in about 100 μ secs. (Nachmansohn, 1969).

6. The Immobilized Enzyme

A. Environment and Action

It has become increasingly apparent over the last decade that the membrane is not just a sac for containing intracellular or intraorganelle constituents, but is in its own right an important structure affecting the enzymic processes in the cell. As well as acting as an organising matrix for enzyme systems such as the electron transport chain, the membrane can have significant effects on the microenvironment at or near the membrane surface. Most biochemical techniques used in enzyme investigation have been based on the solubilized form, free of membrane structures and free in bulk solution. However, an ultimate understanding of biological events *in vivo* can only be gained the closer we get to an *in vivo* situation.

The more obvious areas where membrane enzymes differ from their soluble counterparts are the often more lipophilic nature of the membrane enzymes, lower dielectric constant and the effects caused by surface charge and polyelectrolytes. Coleman (1973) lists several membrane enzymes showing lipid requirements and it is interesting to note that recently, Sihotang(1975),

has shown a marked reactivation of lipid depleted AChE by adding phosphatidyl serine. Also, because the biological membranes contain many polar constituents, they are often highly charged. Contributing to this charge are the phospholipid head-groups, protein side chains, glycolipids and glycoproteins. The charged microenvironment would be very likely to affect the behaviour of any enzymes associated with the membrane.

B. Relationship of Acetylcholinesterase with the membrane

The question of whether AChE is an integral or peripheral membrane protein has already been reviewed earlier in this thesis. Whichever group it falls into, it seems that the active site of the enzyme is oriented towards the outward facing aspect of the membrane at least in the erythrocyte (Coleman, 1973). Silman & Karlin (1967) showed that a membrane preparation of AChE from the electroplax of electric eel showed an anomalous pH dependance. They explained this by saying that the hydrolysis of substrate by AChE generating H^+, caused a pH fall in the unstirred layer around the membrane compared with the bulk solution. Thus, hydrolysis of substrate by the pH-dependant enzyme was decreased. They found that adding buffer to the solution combatted this effect by stabilizing the pH. Also, it was found that using a poor substrate reduced the pH dependant inhibition by lowering the rate of H^+ generation. This anomalous pH dependance is not observed in the solubilized enzyme.

The importance of the membrane to AChE has also been shown recently by the effects of lipid fluidity in the erythrocyte on the allosteric properties of the enzyme. (Bloj, Morero, Farías & Trucco, 1973; Massa, Morero, Bloj & Farías, 1975). When rats were fed corn oil, fatty acid fluidity was high due to the increased levels of unsaturated fatty acids incorporated into the membranes. When lard was fed, the opposite situation applied because of the high level of saturated fatty acids. The erythrocyte bound AChE from rats fed on corn oil showed allosteric behaviour whereas those which were fed lard did not show this phenomenon. Also they showed that insulin abolished the allosteric behaviour in corn oil fed rats and cortisol

induced it in lard fed rats. It was suggested that this was brought about by the decrease and enhancement respectively of membrane fluidity. Farías, Bloj, Morero, Sineriz and Trucco (1975) make the point that it is tempting to stress the importance of the lipid membrane changes on allosteric regulation of AChE in neuronal function. The above observations might suggest certain hydrophobic interaction of AChE with the membrane (Aloni & Livne, 1974) even though many workers feel that it is mainly associated with the membrane by electrostatic interactions because the enzyme can be extracted by high salt concentrations from erythrocyte ghosts. (Mitchell & Hanahan, 1966).

C. Artificially immobilized enzymes

Several methods have been used to immobilize enzymes, such as binding to cyanogen bromide activated Sepharose (Axen, Heilbron & Winter, 1969) or occlusion in polyacrylamide gels (Bernfeld & Wan, 1963). Redwood & Patel, (1974) studied the effect of binding ATPase to phospholipid bilayers in the form of liposomes in order to gain an insight into the mode of binding. Recently, Alsen, Bertram, Gerstcuer & Ohnesorge, (1975) made observations on the effects of binding AChE covalently to polymaleinic anhydride and they found a Km shift as compared to the soluble enzyme. The fact that enzymes can be attached to artificial membranes allows us to gain an insight into the in vivo situation.

D. Electric field near a charged surface

As well as depending on the electric charge at the membrane, the electric field also is related to other factors such as the properties of counter-ions at the membrane surface. Gouy (1910) formulated a model whereby counter-ions represented as point charges are at a uniform charged plane. The number of cations (S_+^c) and anions (S_-^c) near the surface at a point is expressed by a Boltzmann distribution:

$$S_+^c = C_o \exp(-e\psi/kT)$$

and

$$S_-^c = C_o \exp(+e\psi/kT)$$

where ψ is the potential at the point under consideration

C_o is the bulk concentration of each ionic species

e is the electronic charge

k is the Boltzmann constant

T is absolute temperature

Davies & Rideal (1963) showed this for ψ as follows:

$$\psi_G = \frac{2kT}{e} \sin h^{-1}\left[\frac{\sigma}{C_i^{\frac{1}{2}}} \left(\frac{500\pi}{DRT}\right)^{\frac{1}{2}}\right]$$

where σ is the number of surface charges per cm^2

D is the dielectric constant

R is the gas constant

C_i is the univalent electrolyte concentration

If the charge density is taken as the number of ions per cm^2 i.e. 10^{16} per unit area ($Å^2$) and the appropriate numerical constants inserted, this becomes (at 20^oC)

$$\psi = 50{\cdot}4 \sin h^{-1} (134/Åc^{\frac{1}{2}})$$

(Gaines, 1966)

E. The Effect of a Charged Surface on Km (app) of an Immobilized enzyme

As has already been described for cations and anions, charged substrates are distributed between immobilized enzyme and bulk solution depending on the charge of membrane and substrate, (Goldstein, Levin & Katchalski, 1964) These workers have shown that if a Maxwell-Boltzmann distribution is assumed, the substrate concentration near the surface (S_s) is related to the bulk solution concentration (S_o). As follows:

$$S_s = S_o \text{ exp. } (z\, e\, \psi/kT)$$

where z is the charge on the substrate

e is the positive electron charge

ψ is the membrane potential

If z e and ψ are of opposite charge, $S_s > S_o$ whereas if they are of like charge then $S_s < S_o$.

Goldstein et al (1964) have derived the following relationship between the Km (app) for the free solution and immobilized enzyme and the potential ψ :

For the enzyme in free solution

$$V = \frac{V_{max}\ S}{Km(app) + S}$$

thus the velocity (V') of the immobilized enzyme reaction is

$$V' = \frac{V_{max}\ S_o\ \exp\ (z\ e\ \psi\ /kT)}{Km + S_o\ \exp\ (z\ e\ \psi\ /kT)}$$

$$\text{Now,} \quad V' = V_{max}/2$$

$$\text{when} \quad S_o = Km(app)\ \exp\ (-z\ e\ \psi\ /kT)$$

$$\text{Therefore } K'm\ (app) = Km(app)\ \exp\ (-z\ e\ \psi\ /kT)$$

where $K'm(app)$ is the apparent Michaelis constant for the immobilized enzyme.

Therefore

$$\Delta pKm\ (app) = pK'm\ (app) - pKm\ (app)$$

$$= \log_{10} Km\ (app)/K'm\ (app)$$

$$= 0.43\ e\ \psi\ z/kT$$

It can thus be seen that the relationship between the immobilized enzyme and its substrate depends on the charge of both factors.

SECTION II: METHODS

1. Assays

A. Acetylcholinesterase

(i) pH-stat. Assay by pH-stat depends on the continuous and automatic titration of H^+ions liberated by ester hydrolysis (Wilson & Cabib, 1954).

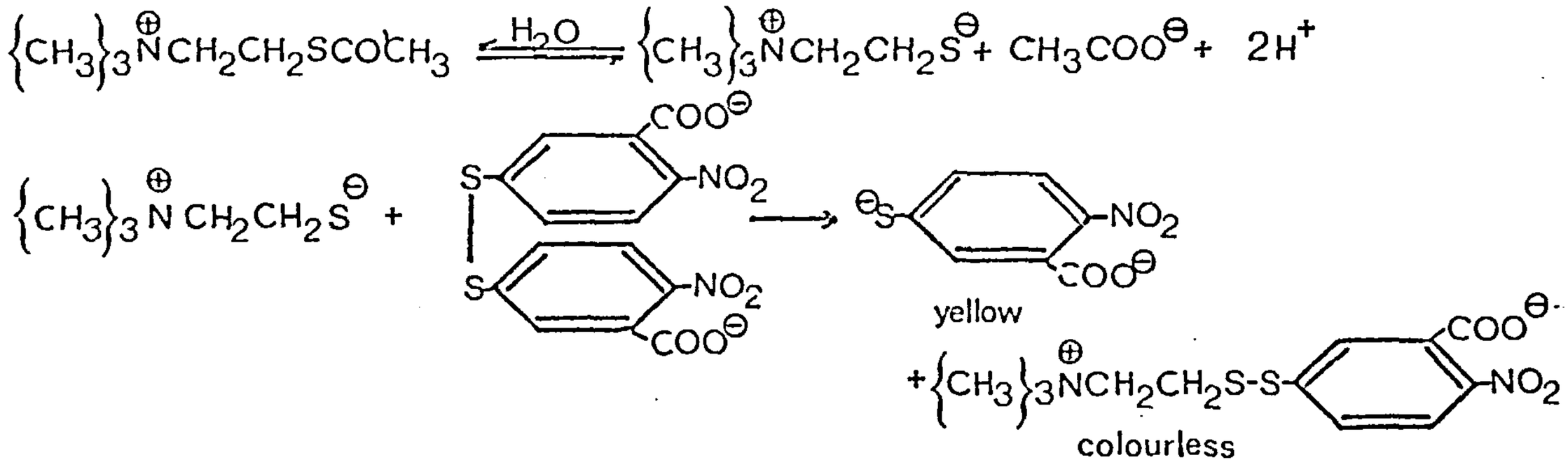
$$CH_3CO.O.CH_2CH_2\overset{\oplus}{N}(CH_3)_3 + H_2O \rightleftharpoons CH_3COO^- + H^+ + HOCH_2CH_2\overset{\oplus}{N}(CH_3)_3$$

The technique is based upon an earlier manual technique whereby a constant pH was maintained by the addition of sodium hydroxide from a manually operated burette (Glick, 1937). In the modern apparatus a glass-calomel electrode system connected to a pH-meter registers the pH of the reaction medium. This in turn automatically directs the addition of sodium hydroxide from a burette into the reaction vessel to keep the pH at a constant predetermined value. The volume of sodium hydroxide consumed is monitored by a pen recorder. No buffer is necessary in the assay medium although with highly active membrane preparations of enzyme it is advisable to have at least a low concentration of buffer present in order to reduce the effects of a pH gradient at the membrane surface (Silman & Karlin, 1967).

AChE was routinely assayed by adding 0.3ml. of enzyme sample to 7.4ml. of 0.15 mol/l. NaCl + 1.3 mmol/l. $MgCl_2$ and measuring the spontaneous release of H^+ions for 5 mins. at 30°C. and pH 7.9. After this 0.3ml. acetylcholine iodide was added to give a final concentration of 1 mmol/l. and the enzyme activity measured for a further 5 mins. When kinetic measurements were made at low concentrations of substrate below 5 µmol/l, a second burette containing 20mM acetylcholine iodide was employed so that a constant substrate was maintained for a sufficiently long time to keep the titration curve rectilinear or only slightly curved. (Jensen-Holm, 1961).

(ii) Ellman spectrophotometric method. The Ellman method (Ellman et al, 1961) is confined to thioester substrates. The mercaptide formed from the hydrolysed thioester reacts with an oxidising agent, 5,5′- dithiobis -

(2-nitrobenzoic acid) (DTNB). The DTNB splits into two products one of which, 5-thio-2-nitrobenzoate, absorbs at 412nm.



Brownson and Watts, (1973) reported that the DTNB interacts with AChE causing a marked activation, and so they recommended the use of 2,2' -dithiodipyridine as the oxidising agent. Augustinsson & Eriksson (1974) however disagree with these findings and advise the continued use of DTNB in the AChE assay.

For routine assay, 50 µl. enzyme was added to 3ml. sodium phosphate buffer (0·1 mol/l, pH 8·0) and incubated at 30°C. for 10 min. then 100 µl. DTNB* (0·01 mol/l) was then added to the medium followed by 20 µl. acetylthiocholine iodide (158·5 mmol/l) to give a final concentration of 1 mmol/l substrate. The increase in absorbance was followed at 412nm. on a Perkin-Elmer SP 124 double beam spectrophotometer.

* The stock DTNB was made up by dissolving 39·6mg. in 10ml. sodium phosphate buffer (0·1 mol/l, pH 7·0) containing 15mg. sodium bicarbonate. The reagent is unstable at more alkaline pH's.

From the extinction coefficient of the chromophore which is $1{\cdot}36 \times 10^4$ Litre.mol^{-1}. cm^{-1} the specific activity of the enzyme can be thus calculated:

$$\frac{\Delta E \times 1000 \times 3{\cdot}17}{\text{min.} \times 1{\cdot}36 \times 10^4 \times 0{\cdot}05} = \frac{\Delta E}{\text{min}} \times 4{\cdot}66 \ \mu\text{mol. min}^{-1}\text{. ml}^{-1}$$

B. Protein

Protein was accurately determined by the method of Lowry et al (1951) using crystalline bovine serum albumin as standard. However if Triton X-100

was present, a gelatinous precipitate formed, but this interference could be overcome by centrifuging the precipitate (1000g. 5 min) and incorporating Triton X-100 in the reagent blank and standards; (Hartree, 1972; ChandraRajan & Klein, 1975).

When many samples were being assayed, protein was estimated by measuring the absorbance at 280nm. providing Triton X-100 was not present. The detergent has a chromophore in the region of 280nm. which interferes with the readings, so a biuret method (Plummer, 1971) was sometimes used as an alternative.

C. Triton X-100

The method of Stevenson (1954) modified by Wright (1971) was used for this assay. Phosphomolybdic acid was added to the detergent and the resulting precipitate dissolved in sulphuric acid. The exinction of this solution was then read at 520nm.

To 2 ml diluted sample was added 75 µl. hydrochloric acid (2 mol/l), 50 µl barium chloride (10% w/v) and 50 µl phosphomolybdic acid (10% w/v); the mixture was shaken thoroughly after each addition and centrifuged at 3000 r.p.m. for 10 min. The supernatant was drained off and the precipitate dissolved in 3ml. concentrated sulphuric acid. After 40 min. incubation at 37°C. the samples were read at 520nm. A linear relationship between extinction and detergent concentration was obtained up to 0·05% Triton X-100 and therefore most samples had to be suitably diluted.

2. Methods of Solubilization

All solubilization procedures were performed on porcine brains obtained on the day of use from Walls Slaughter House, Acton, Middlesex, or from the Co-operative Society Slaughter House, Woolwich, London. The membranes and blood vessels were removed from the surface of the brains and the cortex excised and dispersed in a Waring Blender for 5 min. at 4°C. Centrifugations were carried out on MSE SS65 or SS50 preparative ultracentrifuges using 8 x 50ml. or 10 x 10ml. capacity rotors. The

criterion of solubility was taken as the enzyme remaining in the supernatant after being centrifuged at 100,000g. for 1h.

A. Triton X-100

The method of Ho & Ellman (1969) was followed with slight modifications. A 20% w/v, suspension of brain cortex in sodium phosphate buffer (0·03 mol/l, pH 7·0) was centrifuged at 100,000g. for 1h. The supernatant was decanted and subsequently used as a 'soluble' preparation of enzyme. The pellet was resuspended in buffer to give a protein concentration of 8·0 mg/ml and Triton X-100 added to a final concentration of 1% w/v. The mixture was stirred for 10 min. at room temperature and centrifuged at 100,000g. for 1h. The supernatant was taken as 'Triton solubilized' enzyme.

When a Triton X-100 free extract was required, the detergent was removed according to the method of Ott et al, (1975). The sample was adsorped onto a column (1 x 10cm) of BioGel hydroxylapatite equilibriated with 30 mmol/l sodium phosphate buffer pH 7·0 and washed with the same buffer until no more Triton X-100 appeared in the eluate. The enzyme was then desorped with 200 mmol/l sodium phosphate buffer pH 7·0, then dialysed against the 30 mmol/l sodium phosphate buffer for 12h.

B. Chelating agents

Solubilization with EDTA was accomplished by modifying the methods of Chan et al, (1972a) and Hollunger & Niklasson (1973). A 20% w/v homogenate was prepared in sodium phosphate buffer (0·03 mol/l; pH 7·0) at 4·0°C. and centrifuged for 1h. at 100,000g. The pellet was resuspended to the original volume of buffer containing EDTA (1 mmol/l). The suspension was stirred for 2h. at 4°C. and then centrifuged at 100,000g. The supernatant was removed and the pellet treated a further two times in the same way.

Parallel schemes were also tried in which EGTA was substituted for EDTA and also tetracaine (10^{-5}M) was incorporated with the EDTA; incubations were carried out at 20°C; an 8% w/v homogenate was used.

C. Autolysis

A 20% w/v. homogenate of brain cortex in sodium phosphate buffer (0·03 mol/l; pH 7·0) with 10 µmol/l sodium azide in order to discourage microbial growth was incubated at 37°C. for 24h. The suspension was then centrifuged at 100,000g. for 1h. and the supernatant taken as 'autolysis solubilized' enzyme. Alternatively the incubation was performed before homogenization.

D. Tryptic digestion

The method of Dudai et al (1972), was used with slightly different concentrations of trypsin: 100 ml. of a 20% w/v. brain cortex homogenate in sodium phosphate buffer (0·03 mol/l, pH 7·0) was centrifuged at 100,000g. for 1h. and the pellet resuspended to 100ml. of buffer. The suspension was then treated with 14mg. trypsin in 2ml. water and incubated for 12h. at 25°C. Following this, 14mg. of soybean trypsin inhibitor in 2ml. water was added and the mixture centrifuged for 1h. at 100,000g. The supernatant was taken as 'trypsin solubilized' enzyme.

3. Affinity Chromatography

Synthesis of affinity columns fell into two stages. Firstly, a beaded agarose resin was activated under stringent conditions of pH and temperature. Secondly, an active site ligand specific for AChE was bound to the resin matrix via a spacer arm. For two of the columns, the ligand and spacer arm were synthesised 'in toto' then coupled to the activated agarose. The other column was constructed by consecutively activating the agarose, coupling the spacer arm and then attaching the ligand.

A. Materials

Materials for all three affinity columns were obtained from the following sources. N - Benzyloxycarbonyl - ε - aminocaproic acid and 1 - (3-dimethylaminopropyl) - 3 - ethyl-carbodiimide hydrochloride were purchased from Aldrich Chemical Company Ltd., Wembley, Middlesex. Triethylamine and p-dimethylaminoaniline were supplied by Fisons,

Loughborough, Leics. Anhydrous HBr in glacial acetic acid, isobutyl--chloroformate, iodomethane, 9-chloroacridine and aminopyridine were obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A. Phenol was obtained from Fisons and redistilled before use.

B. ε -Aminocaproyl-PTA-agarose column

i. Preparation of the ligand /N-(ε-Aminocaproyl)-p-aminophenyl/ trimethylammonium bromide hydrobromide. The method of Silman & Dudai (1974) was employed with slight alterations to the procedure (Fig.II.1). The first step was based on the mixed anhydride method of Greenstein & Winitz (1961).

N-Benzyloxycarbonyl-ε-aminocaproic acid (60 mmol; 15·9g.) was dissolved in 200ml. ethyl acetate and cooled with vigorous stirring in a salt-ice bath. Triethylamine (60 mmol; 6·1g.) followed by isobutylchloroformate (60 mmol; 8·2g.) were then added and left for 20 min. p-Dimethylaminoaniline (60 mmol; 8·2g.) was dissolved in 50ml. ice cold ethyl acetate and added drop-wise to the vigorously stirred solution; this was left for a further 5h. at room temperature. The resulting precipitate was filtered, washed with water, ethyl acetate and light petroleum (b.p. 40-60°C.) and dried by suction. The crude material (compound I) was treated with activated charcoal in hot methanol and recrystallized from methanol.

yield of purified N-(N-benzyloxycarbonyl-ε-aminocaproyl)-N′,N′-dimethyl--p-phenylenediamine = 8·23g. 35·8%
m.p.found = 110 - 111°C.
m.p. lit. = 110 - 111°C.

The above compound (22·1 mmol; 8·23g.) was suspended in 40ml. methanol and added to iodomethane (110·5 mmol; 5·26g.) The above mixture was refluxed for 3h. taken to dryness under reduced pressure and triturated with 200ml. diethyl ether. The product, /N-(N-benzyloxycarbonyl-ε-amino--caproyl)-p-amino phenyl/ trimethyl ammonium iodide was recrystallized from 100ml. absolute ethanol (compound II)

yield compound II = 7·64g. 70·7%
m.p.found = 142·5 - 144°C.
m.p. lit. = 143 - 145°C.

Compound II (10 mmol; 5.25g.) was dissolved in 10ml. glacial acetic acid + HBr. This mixture was left at 25°C. for 40 min. and then precipitated with 400ml. dry diethyl ether. Trituration with five batches of dry diethyl ether completed solidification. The solid was dried over NaOH pellets and under reduced pressure for 48h. The dry [N-(ε-aminocaproyl)-p-aminophenyl] trimethyl ammonium bromide hydrobromide, (ε-aminocaproyl-PTA compound III) was recrystallized from 200 ml. absolute ethanol.

yield compound III = 2.95g. 69.3%
m.p.found = 190 - 192°C.
m.p. lit. = 191 - 194°C.

ii. Preparation of ε-aminocaproyl-PTA-agarose. The affinity column was prepared by a method based on that of Axen et al (1967) as modified by Blumberg et al (1970). Cyanogen bromide (7g.) was added to 70ml. water and stirred for 10 min. during which time most of it dissolved. A Sepharose 4B slurry was washed with water and 70ml. of this added to the stirred cyanogen bromide-water mixture. The pH was immediately adjusted to 11 with 6 mol/l - NaOH and the mixture cooled below 20°C. with crushed ice for 8 min. The activated gel was then rapidly washed with 1000ml. water at 4°C. on a Buchner funnel and the wet Sepharose 4B added quickly to a solution of ε-aminocaproyl-PTA (93 μmol; 50mg.) in 30ml. $NaHCO_3$ (0.5 mol/l). This mixture was shaken gently (not mechanically stirred) for 16h. at 4°C, then filtered and washed thoroughly with $NaHCO_3$ (0.1 mol/l) and water. The amount of ligand coupled was found by estimating spectro-photometrically the quantity of ligand remaining in the washings. Approximately 1 μmol. ligand was coupled per ml. resin.

At pH 9.8, ε-aminocaproyl-PTA λ max = 245 nm
ϵ = 13,430 litre. mol^{-1} cm^{-1}

The affinity resin was washed extensively before use with the elution buffer.

C. MAC-agarose column

i. Preparation of the ligand (1-methyl-9-/N$^{\beta}$-ϵ-aminocaproyl)-β-aminopropylamino/ acridinium bromide hydrobromide. The liquid was synthesised by the method of Silman & Dudai (1974) with some amendments advised by Silman (personal communication). See Fig.II.2.

Phenol (~~0·78~~ 1·26 mol; 120g.) was heated to 70°C. and 9-chloroacridine (93·60 mmol; 20g.) added to the melt. After all the solid had dissolved 100ml. 1,2-propylenediamine was added to the vigorously stirred mixture and the temperature raised to 120°C. It was critical that this temperature was not exceeded. After 30 min. the product 9-(β-aminopropylamino) acridine (compound IV) was precipitated by pouring the reaction mixture with rapid stirring into 1600ml. of NaOH (0·75 mol/l). This was left overnight to complete the solidification, filtered and washed with NaOH (2 mol/l) followed by water. The crude product was dried in vacuo then refluxed in 1100ml. absolute benzene and filtered. The insoluble residue was discarded and the filtrate concentrated to 200ml. Then 50ml. petroleum was added to the filtrate and left at 4°C. overnight. The crystalline product was filtered and washed with benzene.

yield of 9-(β-aminopropylamino) acridine (compound IV) = 9·5g. 39·9%
m.p.found = 131 - 139°C.
m.p. lit. = 131 - 133°C.

The mixed anhydride method of Greenstein & Winitz (1961) was employed for the next step. N-Benzyloxycarbonyl-ϵ-aminocaproic acid (30 mmol; 7·9g) was dissolved in 150ml. dry ethyl acetate in a salt-ice bath at -10°C. and stirred vigorously. To this mixture was added triethylamine (30 mmol; 3·05g) followed by isobutylchloroformate (30 mmol; 4·1g) and this was left stirring for 20 min. at -10°C. The reaction mixture was filtered by suction and the precipitated triethylamine hydrochloride washed with 20ml. dry ethyl acetate. The combined filtrate and washings was returned to the salt-ice bath and a solution of 9-(β-aminopropylamino) acridine (25 mmol; 6·3g.) dissolved in dry dimethylformamide (100ml.) at -10°C. was added. This was left for a further 20 min. at this temperature and then for 12h. at

room temperature. Thin layer chromotography on silica gel in glacial acetic acid indicated that there had been total coupling to give compound V. The reaction mixture was evaporated to dryness under reduced pressure and the residue dissolved in 50ml. absolute methanol. Then 8ml. iodomethane was added to the solution, refluxed for 4h. and left overnight at room temperature. Silica gel thin layer chromotography in ethyl acetate showed that quaternization had gone to completion. The solution was evaporated dry under reduced pressure, extracted twice with 100ml. aliquots of dry ethyl acetate and the product recrystallized from 80ml. 2-propanol. The solid compound VI, [N - (N-benzyloxycarbonyl-ε--aminocaproyl)-β-aminopropylamino]acridinium iodide was washed with ice-cold 2-propanol followed by ice cold diethyl ether.

yield compound VI = 7·2g.
m.p.found = 155 - 156·5°C.
m.p. lit. = 156 - 157°C.

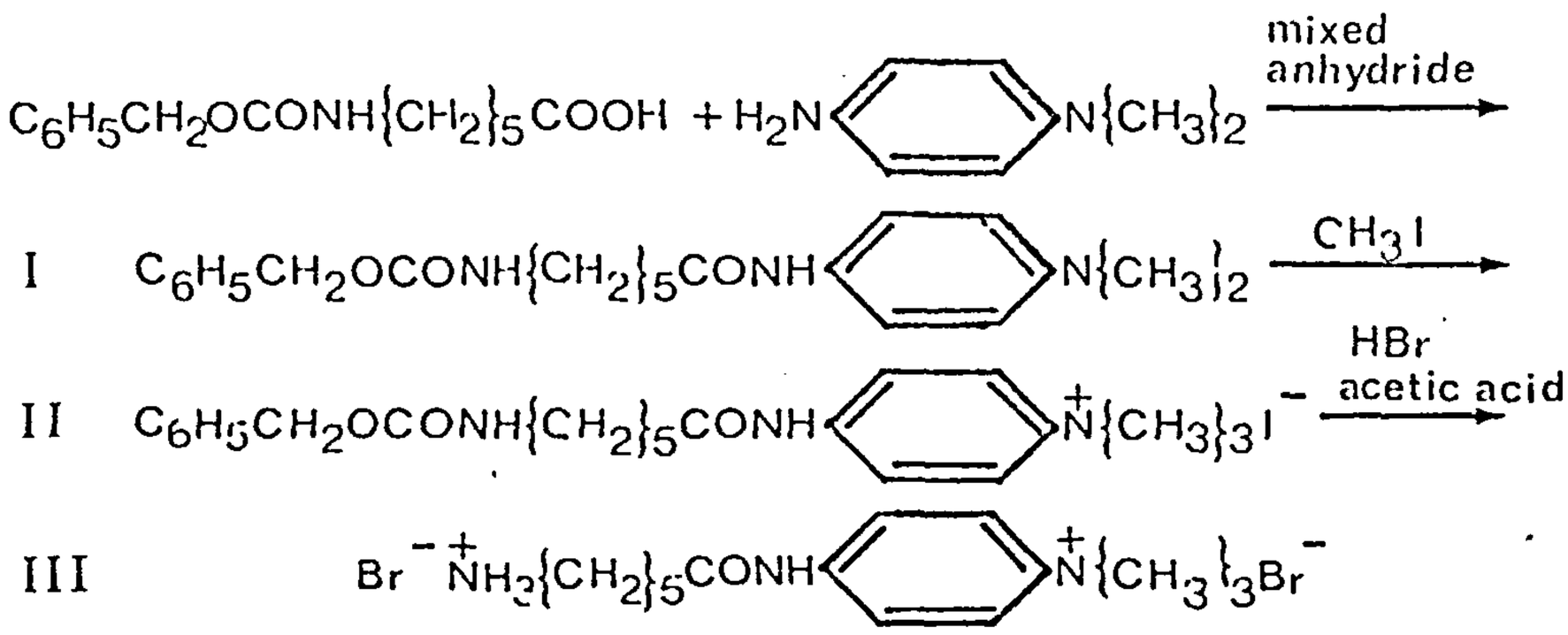
The above quaternary compound VI (6·4 mmol; 4·1g.) was dissolved in 40ml. anhydrous glacial acetic acid and then 80ml. anhydrous HBr in glacial acetic acid was added. This solution was left for 30 min. at room temperature and the product precipitated with dry diethyl ether. The precipitate was triturated with 5 batches of diethyl ether until it solidified. The solid was filtered, washed with diethyl ether and left for 24h. under reduced pressure over dry NaOH pellets. The resulting crystals of (1-methyl-9-[N^β-ε-aminocaproyl)-β-aminopropylamino] acridinium bromide hydrobromide, (MAC) were recrystallized from absolute ethanol.

yield of MAC = 2·5g (72%)
m.p.found = 236 - 240°C.
m.p. lit. = > 240°C with decomposition

ii. Preparation of MAC-agarose. The ligand was coupled to the Sepharose 4B in the same way as described for the preparation of the ε-aminocaproyl--PTA-agarose resin. MAC (0.09 mmol. 50mg.) was mixed with 70ml. of the cyanogen bromide activated Sepharose 4B. By spectrophotometric assay of the washings, between 0.5 - 1.0 μmol. MAC were found to be coupled per ml. resin.

Fig. II.1

SYNTHESIS OF ε-AMINOCAPROYL-PTA.



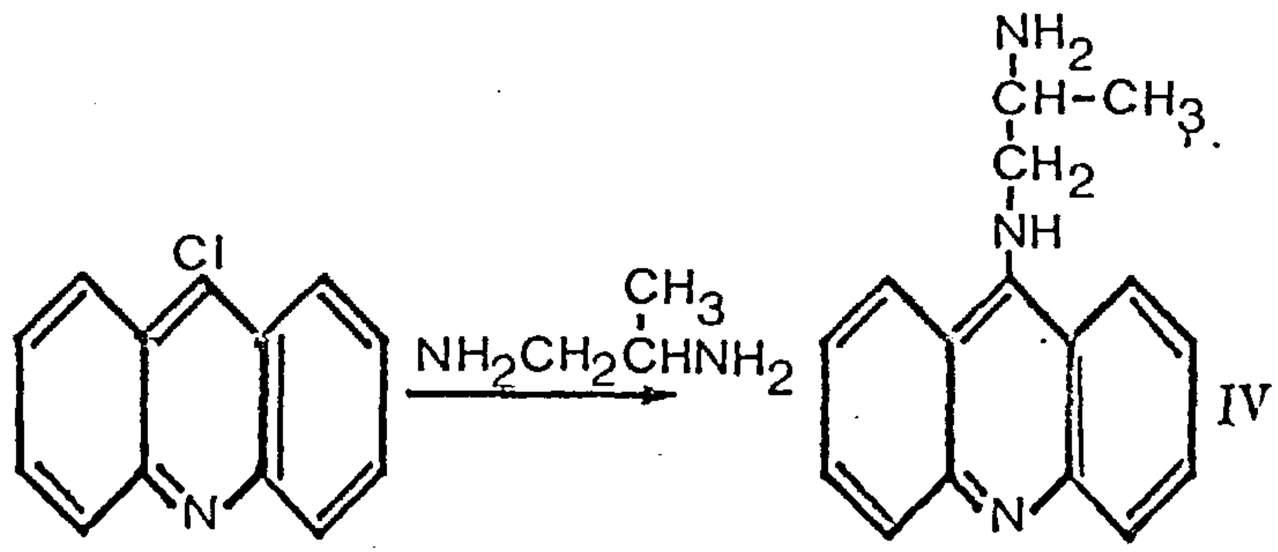
I $C_6H_5CH_2OCONH(CH_2)_5CONH$–PTA–$N(CH_3)_2$

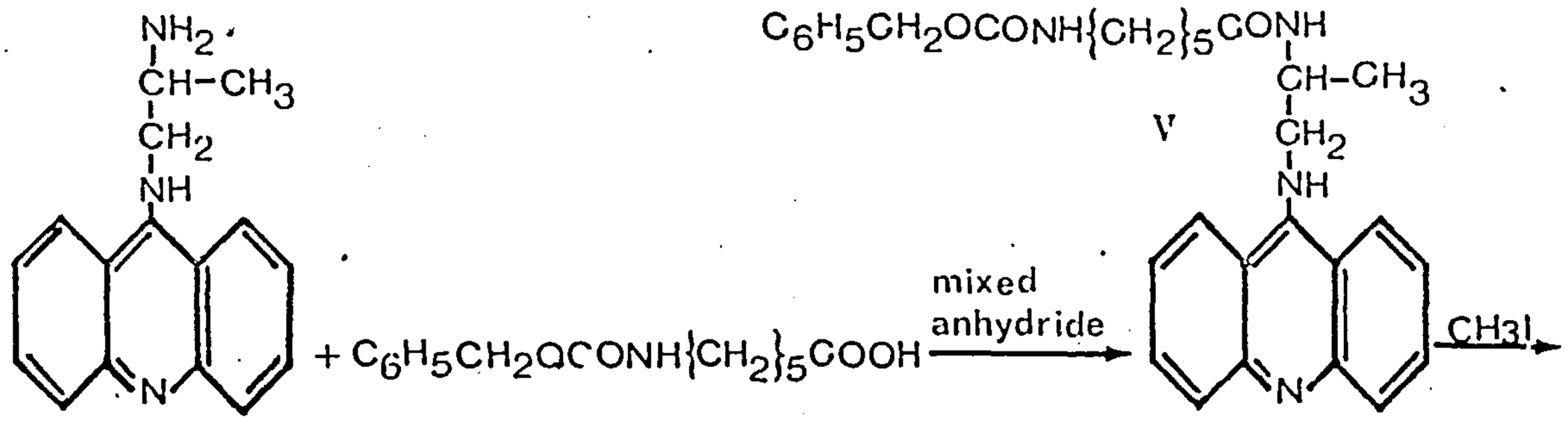
II $C_6H_5CH_2OCONH(CH_2)_5CONH$–PTA–$\overset{+}{N}(CH_3)_3\,I^-$

III $Br^-\overset{+}{N}H_3(CH_2)_5CONH$–PTA–$\overset{+}{N}(CH_3)_3\,Br^-$

Fig II.2

SYNTHESIS OF MAC.





At pH 8·3, MAC λ max = 393nm; 410nm; 431nm;
ϵ = 7,880; 12,050; 10,150

D. MAP-agarose column

i. Preparation of the ligand N-methyl- 3 - aminopyridinium iodide.

Aminopyridine (53 mmol; 5g.) dissolved in 75ml. acetone was added to iodomethane (292 mmol; 15ml.) and stirred for 18h. The precipitated N-methyl - 3 - aminopyridinium iodide (MAP) was filtered and washed with acetone. Fig.II.3.

Yield MAP = 11·2g (95%)
m.p = 120 - 122°C.

ii. Preparation of MAP-agarose

The affinity resin was built up step-wise by a method based on the procedures used by Berman & Young (1971), and Goodkin & Howard (1974).

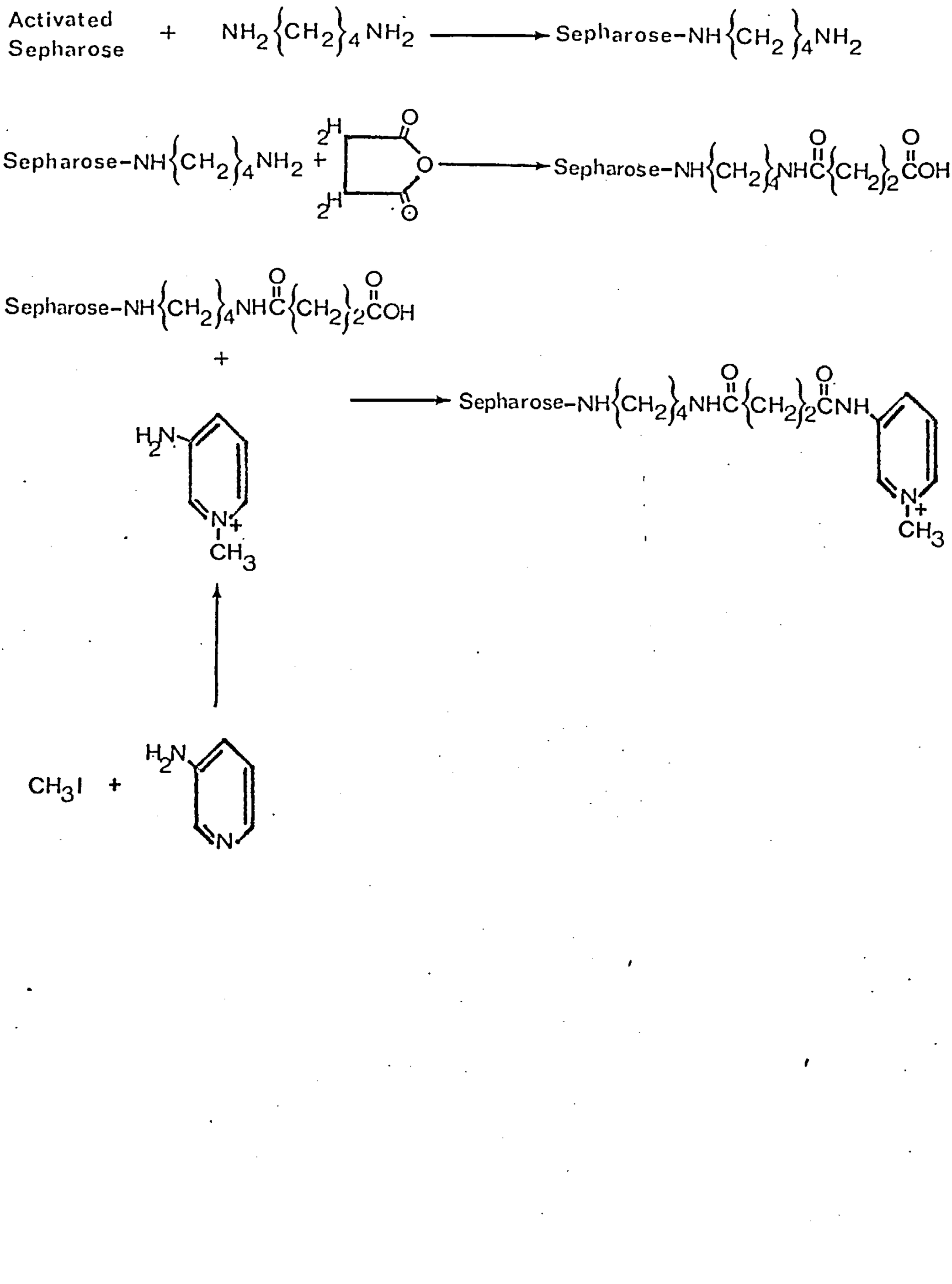
100ml. washed Sepharose 2B was suspended in 100ml. water. Cyanogen bromide (30g.) was added to the stirred medium and the temperature kept below 20°C. with crushed ice and pH at 11·0 with NaOH (4 mol/l). After 12 min. the suspension was filtered rapidly under suction with 1000ml. sodium borate buffer. (0·1 mol/l; pH 9·5) at 5·0°C. Diaminobutane (198 mmol; 17·4g.) in 100ml. sodium borate buffer (0·1 mol/l; pH 9·5) was immediately added and the mixture shaken gently at 4·0°C. overnight.

The excess amine was removed by filtration on a sintered glass funnel and the washed resin dispersed in 100ml. water. Succinic anhydride (100 mmol; 10g.) dissolved in 105 ml. water at 4·0°C. was added to the resin and the pH raised to 6·0 with NaOH (4 mol/l). After the pH had become stable, the suspension was left for 5h. at 4·0°C.

N-Methyl -3 -aminopyridinium iodide (0·6 mmol; 0·13g.) was added to 6·0 ml. succinylated resin in the presence of 1-(3-dimethylaminopropyl)-3--ethyl carbodiimide hydrochloride (4·5 mmol; 0·86g.). The reaction mixture was then shaken gently overnight at 4°C. then thoroughly washed before use with elution buffer.

Fig.II.3

SYNTHESIS OF MAP--AGAROSE



E. Conditions for elution.

Fundamentally, the buffer used for elution was sodium phosphate buffer (0·03 mol/l; pH 7·0) which was run through columns with a bed volume of 12ml. The enzyme was run through the resin at a rate of 30-40 ml/h. after which the column was washed with 5-10 column volumes of buffer. The enzyme was then eluted with 5 column volumes of elution buffer containing the AChE inhibitors edrophonium chloride (10 mmol/l) or decamethonium bromide (10 mmol/l) and fractions of 2-5ml. collected. Finally the column was washed with 5 column volumes of buffer containing NaCl (1 mol/l) followed by guanidine hydrochloride (6 mol/l) and then 50 column volumes elution buffer. The peak of enzyme activity was dialysed against three changes of 2000ml. elution buffer over 72h. The effect of incorporating 1% - Triton X-100 and NaCl (0·1 mol/l) in the elution buffer was also tested. All operations were conducted at $4^{o}C$.

The above elution programme was essentially based on procedures followed by Dudai *et al* (1972a); Dudai *et al* (1972b); Berman & Young (1971); Goodkin & Howard (1974), with extensive modifications.

4. Electrophoresis

A. Polyacrylamide rods. Ornstein, (1964); Davis, (1964)

Stock solutions were prepared as follows:

Solution A

1 mol/l - HCl	48ml
Tris	36·6g
TEMED	0·23ml
Water to	100ml
pH 8·9	

Solution B

1 mol/l - HCl	48ml
Tris	5·98g
TEMED	0·46ml
Water to	100ml
pH 6·9	
Adjust with 1 mol/l - HCl	

Solution C

Acrylamide	28·0g
Bis	0·735g
Water to	100ml

Solution D

Acrylamide	10·0g
Bis	2·5g
Water to	100ml

Solution E

Riboflavin	4mg
Water to	100ml

Solution F

Sucrose	40g
Water to	100ml

Working solutions

Small Pore solution 1.

1 part A
2 parts C
1 part water

Small pore solution 2.

Ammonium persulphate	0·14g
Water to	100ml

Large pore solution

1 part B
2 parts D
1 part E
4 parts F
pH 6·7

Stock buffer solution

Tris	6·0g
Glycine	28·8g
Water to 1 litre	
pH 8·3	

Equal volumes of small pore solutions 1 and 2 were mixed, degassed and aliquots of 0·9ml. added to eight electrophoresis tubes. A water overlay was applied to the surface of the solutions and these were left to polymerise (20-40min.) The water overlays were removed and 0.15ml. degassed large pore solution syringed on top of the gels. The solutions were overlaid with water and the tubes placed in a fluorescent light until polymerisation was complete (20-40min.) A mixture of 150 µl. large pore solution and 10-40 µl. sample protein was added to each tube and polymerised as described above.

The stock electrophoresis buffer was diluted x10 with water and poured in the anodic and cathodic reservoirs of a Quickfit PAGE apparatus. Eight gels were run per apparatus at a constant current of 2·5 mA per tube for 1½ - 3h. at 4°C.

Protein was stained by placing the gels in Coomassie Brilliant Blue stain (0·2% w/v) in methanol:acetic acid:water in the ratios 5:1:5 for 24h. For destaining, the gels were incubated in acetic acid (7% v/v) for periods long enough to remove the background stain.

AChE was stained by the method of Koelle (1951) as notified by Lewis & Shute (1966). Acetylthiocholine iodide (100 mg.) was dissolved in 8ml. water and 14ml. cupric acetate was added drop-wise to the stirred solution. This was then centrifuged at 2000 rpm. for 10 min. and the supernatant decanted into a beaker containing glycine (60 mg.). Sodium acetate (2 mol/l) was added to the solution to give a final pH between 6.5 and 7·0. Gels were incubated in this stain for 6-24h. and destained in 7% v/v acetic acid.

When the specific anticholinesterase BW 284 C51 dibromide had been used to inhibit the enzyme, the gels were incubated in a solution of the inhibitor (10 µmol/l) for 1h. prior to staining.

B. Polyacrylamide slabs.

Ready made 'Gradipore' polyacrylamide gradient gels (4-24%) were used (Universal Scientific Ltd., London), or were made up using the 'Gradipore'

gradient former and gel casting set (McIntosh, 1973). The gel casting unit was especially used when Triton X-100 was incorporated in the slabs.

Tris-borate-EDTA buffer solution (pH 8·3) was made up as follows:

EDTA	4·65g	2·5	mmol/l
Tris	53·75g	88·74	,
Boric acid	25·20g	81·51	,
Water to 5 l.			

Gels were pre-electrophoresed without samples in a three cell 'Gradipore' electrophoresis unit at 100v. until the current had fallen to a steady value between 30-35mA. Samples (30 µl.) were applied to the top of the gels in a plastic spacer which allowed 14 samples to be run per gel. The samples were overlaid with buffer and electrophoresis performed for 24h. at 100v. and room temperature. The buffer was circulated through a heat exchanger immersed in a water bath to keep the temperature stable.

Staining of the gels for protein and enzyme was accomplished by removing them from the glass retaining plates and treating them in the same way as polyacrylamide rods.

Each batch of gels was standardized by electrophoresing several proteins of known molecular weight and plotting migration distance against $\log_{10}$ molecular weight. The proteins employed were haemaglobin, lactate dehydrogenase, alcohol dehydrogenase, bovine serum albumin, caeruloplasmin, horse spleen apoferritin and thyroglobulin.

C. Starch block electrophoresis

Although this method is too laborious as a routine analytical tool, it has its uses for semi-preparative procedures.

Potato starch was obtained from British Drug House, Poole, Dorset. This was washed twice with water and then twice with sodium phosphate Buffer (0·1 mol/l; pH 8·0). Triton X-100 (1%) was incorporated in the buffer if the sample to be electrophoresed contained the detergent. Excess buffer was decanted and the starch pressed into a block with the aid of perspex formers 19cm.x 12cm.x 1cm. or 30cm.x 10cm.x 1cm. Wicks consisting of several layers of muslin connected the block with the buffer in

the electrode tanks. Anode and cathode tanks were each divided into two sections connected by cotton wool plugs. This arrangement prevented pH changes around the electrodes affecting the buffer in contact with the block. The block was pre-electrophoresed at 6-7 v/cm. for 1h. at 4^{o}C. The enzyme sample containing 5-10mg. protein was applied to a laterally cut groove, 0·5cm. wide and one third of the distance along the block from the cathode. A mixture of haemaglobin bovine serum albumin and bromophenol blue was applied as markers to a cavity in line with the groove. The cavity and groove were filled with starch and electrophoresis conducted at 6-7 v/cm. for times up to 18h.

The final positions of markers were noted and the longitudinal section containing them discarded. The remaining block was cut transversely into strips of 0·5cm. width between the markers and 1cm. width either side of the markers. Each portion of starch was eluted twice through a sintered glass funnel with 2ml. aliquots of buffer. The AChE was assayed by the Ellman method and the electrophoresis patterns presented as histograms showing total activity in each strip cut from the block.

5. Density gradient centrifugation

In order to determine the sedimentation coefficient and approximate molecular weight of AChE, sucrose gradients were used in the swing-out rotors of a preparative ultracentrifuge. This was possible by determining the ratio of mobilities between AChE and a standard well characterized protein. (Martin & Ames, 1961).

A Preparation of gradients

Sucrose gradients were prepared in 25ml. or 5ml. capacity polycarbonate tubes. For the 25ml. tubes a modification of the apparatus employed by Britten & Roberts, (1960), was used. Two vertical chambers were connected at the bottom by a polystyrene tube and another tube also extended from the bottom of one of the chambers. An overhead stirrer projected into the chamber with the outflow tube. A solution of 9ml. sucrose (20% w/v) in sodium phosphate buffer (0·03 mol/l; pH 7·0) was added to the chamber

housing the stirrer and 9ml. sucrose (5% w/v) in the same buffer added to the other chamber. The apparatus was rocked back and forth to remove air bubbles from the interconnecting tube and the outflow tube positioned in a 25ml. centrifuge tube containing 2ml. buffered sucrose (60% w/v). The nozzle of the outflow tube was adjusted so that it just penetrated the meniscus of the sucrose. The overhead stirrer was switched on and the stop-cock regulated so that the sucrose flowed into the tube at approximately 2 ml/min. Meanwhile the outlet nozzle was maintained at just below the meniscus.

When 5ml. tubes were used, the sucrose was added in equal layers of diminishing concentration. The bottom layer of sucrose was 60% w/v. and on top of this was pipetted ten equal volumes (0·4ml.) of buffered sucrose starting with 20% w/v. diminishing finally to 5% w/v. The tube was then rotated gently back and forth around its longitudinal axis to disperse the interfaces and thus obtain a homogeneous linear gradient. Gradients were stored at 4^{o}C. for 5h. before use.

B. Centrifugation

The standard protein, bovine catalase, which was assumed to have a sedimentation coefficient of 11·4 S. and mol.wt.240,000 (Sumner & Gralén, 1938), was mixed with the enzyme sample and layered by pipette on to the gradients. It was essential that not more than 50mg. protein was applied to each gradient as overloading causes loss of Gaussian shape of the migrating protein zone (Steensgaard et al, 1975).

After spinning for 100,000g. for 17h., the tubes were fractionated and aliquots removed with an MSE tube piercer. Fractions of 0·5ml. were taken from the 25ml. tubes and 3 drops from the 5ml. tubes, then assayed for AChE by the Ellman method. Catalase was assayed by following the decrease in absorbance at 240nm. of a mixture containing 3ml. sodium phosphate buffer (10 mmol/l; pH 7·5), 20μl. hydrogen peroxide (0·9 mol/l) and 20 μl. enzyme sample. Activities were calculated in terms of change in absorbance per minute.

The ratio 'R' was determined experimentally according to Martin & Ames (1961):

$$R = \frac{\text{distance travelled from meniscus by unknown}}{\text{distance travelled from meniscus by standard}}$$

As molecules move at an almost uniform rate:

$$R = \frac{S_{20,w}^{0\cdot725}\ \text{unknown}}{S_{20,w}^{0\cdot725}\ \text{standard}}$$

where $S_{20,w}^{0\cdot725}$ = sedimentation constant extrapolated to the standard state taken as that of water and partial specific volume 0·725 $cm.^3\ g^{-1}$. As most proteins have partial specific volumes in the range 0·70 - 0·75 $cm.^3\ g^{-1}$, the above assumption resulted in less than 3% error in the estimation of $S_{20,w}$.

So, for molecules of the same partial specific volume:

$$R = \frac{S_{20,w}\ \text{unknown}}{S_{20,w}\ \text{standard}}$$

A crude estimation of mol.wt. was obtained:

$$\frac{S_1}{S_2} = \left(\frac{\text{Mol.wt}_1}{\text{Mol.wt}_2}\right)^{\frac{2}{3}}$$

since for most proteins S_1/S_2 is equal to R. (Schachman, 1959).

In order to test the linearity of the gradients, some centrifugations were performed in the absence of samples. The sucrose concentrations were then determined by refractometer and plotted graphically against fraction number.

Also, the efficacy of this technique was tested by running three standard proteins on gradients and comparing their sedimentation coefficients with those found by other techniques. The proteins used were yeast alcohol dehydrogenase, egg white muramidase and beef liver catalase. Assays were performed according to Martin & Ames (1961). Sedimentation values were assumed to be those quoted by Martin & Ames: catalase, 11·4S; muramidase, 2·1S; A.D.H, 7·6S.

Fig. II 1

Gradient former

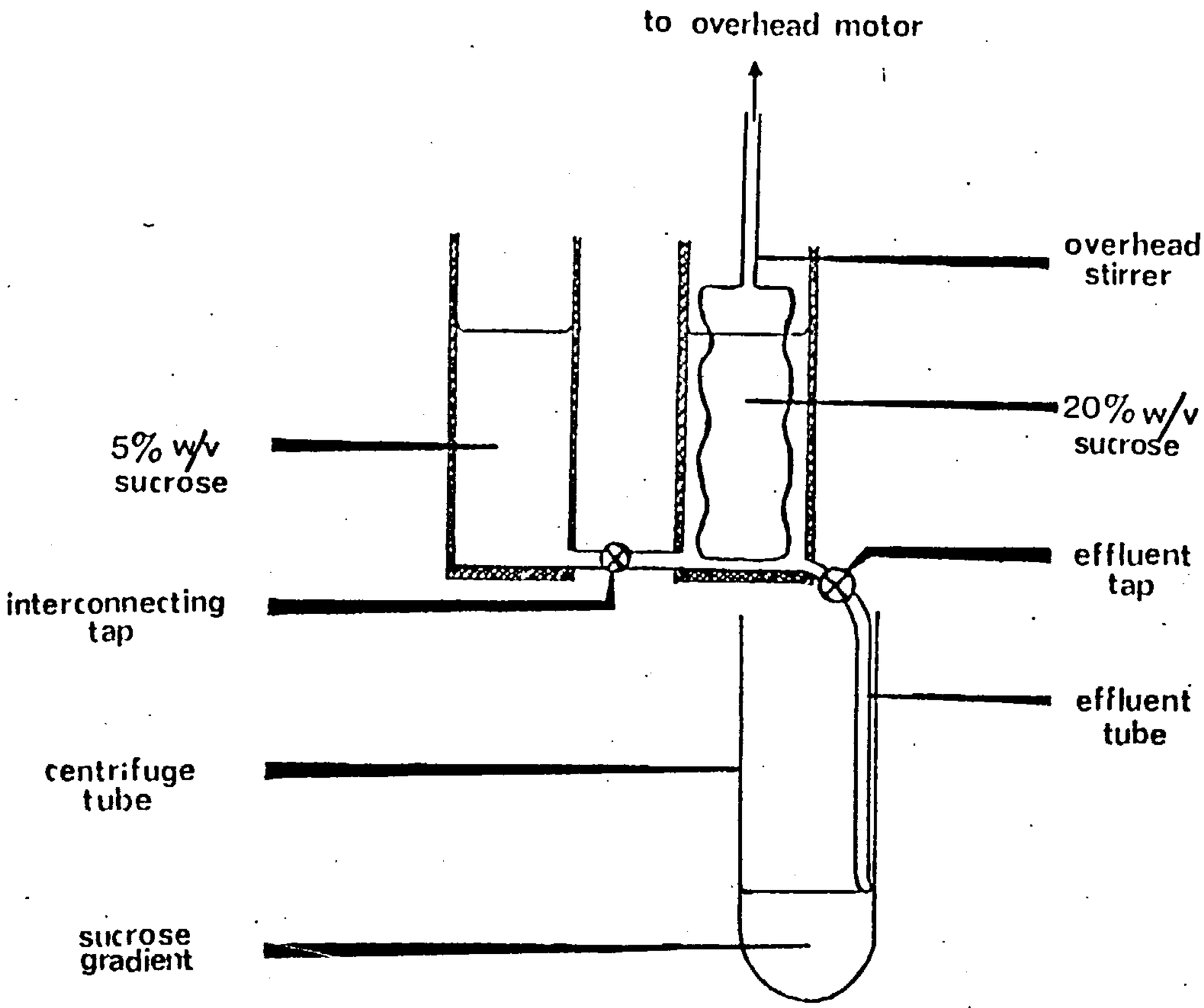
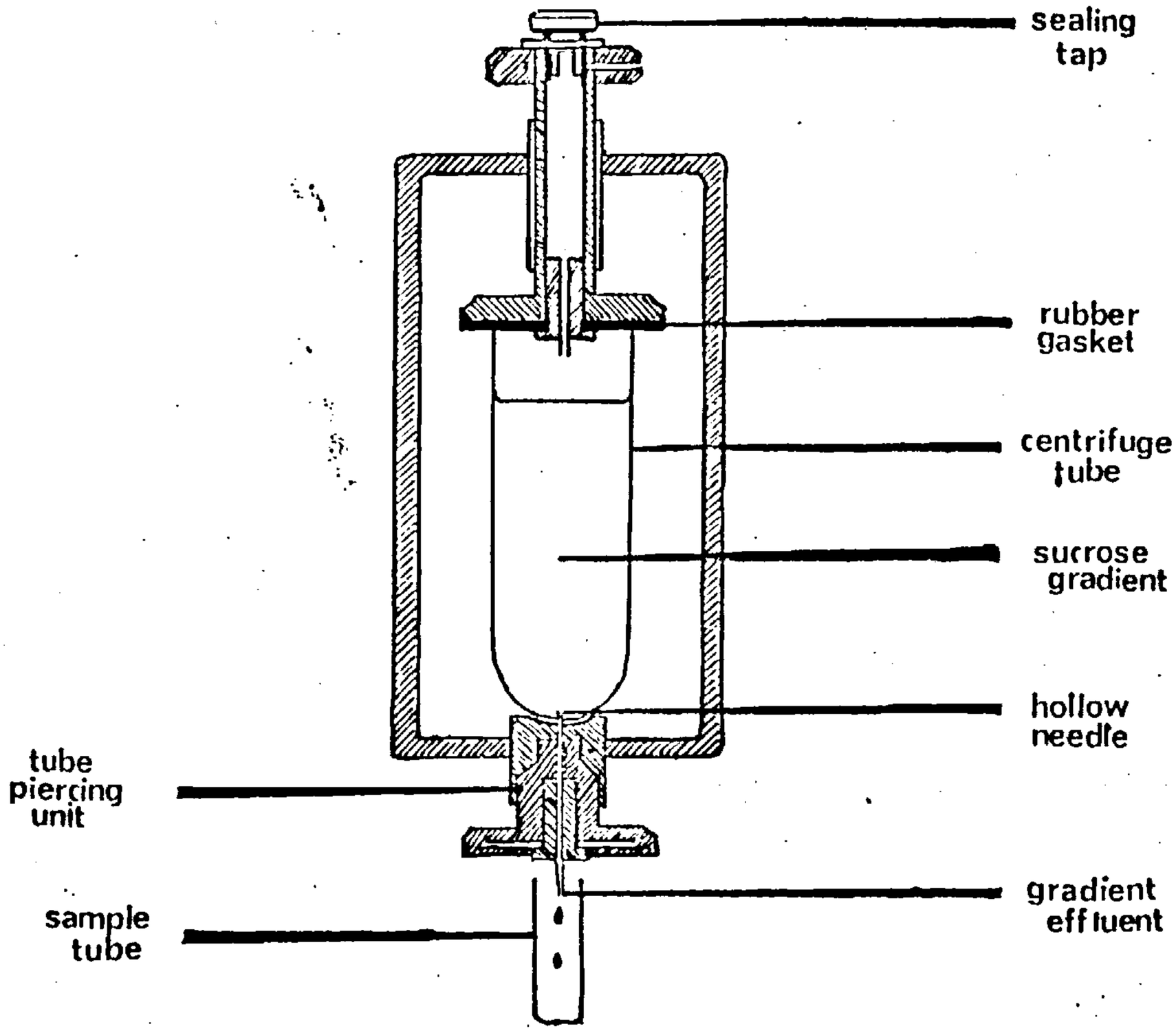


Fig. II 2

M.S.E. Tube piercer



6. Liposomes

A. Preparation of liposomes

Chloroform was evaporated away from the phospholipids under reduced pressure and a cold solution of 4ml. sodium phosphate buffer (0·05 mol/l; pH 7·4) added with several glass balls. (See results section for the composition of phospholipids). The solution was flushed with nitrogen and agitated for 10 min. during which time the phospholipid vesiculated to form liposomes.

B. Formation of AChE-liposome complex and its assay

An aliquot of 0·2ml. liposomes was added to 0·8ml. sodium phosphate buffer (0·05 mol/l; pH 7·4) followed by 0·1ml. DTNB (made up as already described for the Ellman method). Then 5µl. AChE purified by affinity chromotography was added, thoroughly mixed and incubated at 25°C. for 1h. The enzyme reaction was started by adding acetylthiocholine iodide in varying concentrations and measuring the absorbance for 10 min. on a Perkin-Elmer SP.256 dual wavelength spectrophotometer. In order to reduce artefacts due to light-scattering, the beam was selected in the dual wavelength mode. Sample wavelength was set at 412nm. and reference wavelength at 452nm.

C. Binding studies

The procedure was based on that of Redwood & Patel, 1974; Liposomes were made up as above and 1 ml. aliquots (approx. 2µ mol) incubated with 1·0 - 20 µl. AChE purified by affinity chromotography in 7·0ml. sodium phosphate buffer (0·05 mol/l; pH 7·4), for 1h. at 4°C. or 25°C. The liposomes were then sedimented at 100,000g. for 40min. in a centrifuge and resuspended in 1·0ml. buffer. The supernatants and suspensions were assayed for AChE as above and the activity bound per µ mol. phospholipid calculated. Results were represented as Scatchard plots from which could be determined the Kass, for binding, (Scatchard, 1949).

SECTION III: RESULTS

1. Isolation

A. Solubilization

(i) Dilute buffer or water. The enzyme was considered to be soluble if it remained in the supernatant at 100,000g. for 1h. Extraction of a 20% homogenate of brain with water or 0·03 mol/l sodium phosphate buffer (pH 7·0) showed that 13% to 15% of the AChE was soluble; this fraction was referred to as the 'soluble' enzyme (Table III.1). The remainder of activity (85-87%) was sedimented after centrifuging for 1h. at 100,000g. and was therefore assumed to be bound to a particulate fraction (McIntosh, 1973) Further extraction of the pellet obtained from the 100,000g. centrifugation did not result in any further solubilization of enzyme.

(ii) Chelating Agents Repeated homogenization and incubation of brain cortex with EDTA (1 mmol/l) brought nearly half the AChE activity into solubilization (see Table III.2). The yield was not improved by changing the composition of the medium such as decreasing the percentage w/v of brain homogenate or incorporating tetracaine (10^{-5}mol/l) into the medium. Extraction with EGTA (10^{-3}mol/l) solubilized very little of the particulate AChE activity (Table III.3).

(iii) Autolysis Autolysis of a brain homogenate for 24h. did not solubilize a significant amount of AChE and well over 70% of the total homogenate activity was denatured by the treatment (Table III.4). The method was slightly improved when the brain cortex was incubated at 37°C. before homogenization, (Table III.5) but large amounts of the enzyme were still found to have been denatured.

(iv) Tryptic Digestion Incubation of brain homogenates with the peptidase trypsin for 12h. gave variable results. Generally however, very little AChE was solubilized by this method and there was also a small loss of activity. If the homogenate was exposed to trypsin for periods longer than 12h. there resulted a greater degree of inactivation of AChE.

Table III.1

Acetylcholinesterase in the Soluble Fraction

Fraction	Yield of activity (%)	Yield of protein (%)	Specific activity $\mu mol.min.^{-1} mg.^{-1}$
Homogenate(20%)	100	100	0·082
100,000g supernatant	13	37	0·028

Average of 5 experiments

Table III.2

Solubilization of Acetylcholinesterase by repeated homogenization and incubation of brain cortex in phosphate buffer (0·03 mol/l pH 7·0) containing 1 mmol/l EDTA

Fraction	Yield of activity %	Yield of protein %	Specific activity $\mu mol.min.^{-1}mg.^{-1}$
Homogenate(20%)	100	100	0·057
100,000g supernatant from 1st EDTA incubation	14	14	0·060
100,000g supernatant from 2nd EDTA incubation	25	6	0·244
100,000g supernatant from 3rd EDTA incubation	10	2	0·331
Pooled supernatants from EDTA incubations	49	22	0·139

Table III.3

Total Solubilization of Acetylcholinesterase from three incubations and Homogenizations of Brain Cortex under Various Conditions

Fraction	Yield of activity %	Yield of protein %	Specific activity $\mu mol.min^{-1}mg^{-1}$
8% homogenate in buffer + EDTA(10^{-3}mol/l)	100	100	0·016
pooled supernatants	35	15	0·037
8% homogenate in buffer + EDTA(10^{-3}mol/l) and tetracaine (10^{-5}mol/l)	100	100	0·016
pooled supernatants	52	25	0·032
20% homogenate in buffer + EGTA(10^{-3}mol/l)	100	100	0·059
pooled supernatants	16	21	0·046

Average 2-3 experiments

Table III. 4

Solubilization of Acetylcholinesterase by Autolysis of Homogenate

Fraction	Yield of activity %	Yield of protein %	Specific activity $\mu mol.min.^{-1}mg.^{-1}$
Homogenate(20%)	100	100	0.084
Resuspended pellet incubated 37°C. 24h.	29	98	0.025
100,000g. supernatant	1	23	0.004

Average of 3 experiments

Table III. 5

Solubilization of Acetylcholinesterase by Autolysis of Brain Cortex before Homogenization

Fraction	Yield of activity %	Yield of protein %	Specific activity $\mu mol.min.^{-1}mg.^{-1}$
Cortex incubated 37°C.24h. then homogenized(20%)	100 (40)	100 (99)	0.036
100,000g.supernatant.	10 (4)	25 (24)	0.016

Average of 3 experiments

The figures in brackets(Table III.5) give percentage yields assuming that 100% activity is represented by the 20%-homogenate in Table III.4. Thus results in Table III.4.can be directly compared with those in Table III.5.

Table III.6

Solubilization of Acetylcholinesterase by Triton X-100(1%)

Fraction	Yield of activity (%)	Yield of protein (%)	Specific activity $\mu mol.min.^{-1} mg.^{-1}$
Homogenate(20%)	100	100	0·082
100,000g. Pellet	86	58	0·121
100,000g.Supernatant from Triton X-100 treatment of resuspended pellet	56	34	0·141

Average of 5 experiments

(v) Triton X-100 Extraction with Triton X-100 (1% w/v) solubilized 56% of the total homogenate activity (Table III.6). When the 'Triton solubilized' fraction was summated with the 'soluble' fraction (14%) the activity remaining in the 100,000 pellet was consistently found to be 30% of the total homogenate activity.

It was found that there was no difference in enzyme activity between the homogenate with or without 1% w/v Triton X-100 being incorporated. The 56% activity found in the Triton solubilized fraction was therefore assumed to be a true solubilized fraction and not an activated fraction of enzyme activity.

B. Purification by Affinity Chromatography

As it was important to find the optimal elution conditions for purification, various elution media were tried for the three affinity columns. Triton X-100 solubilized AChE was used for all elution procedures as this was found to be the quickest and most efficient way of solubilizing the enzyme.

(i) MAP-agarose column The enzyme (1·0ml) which contained approximately 25mg. protein was applied to the column previously equilibriated with sodium phosphate buffer (0·03 mol/l, pH 7·0) containing Triton X-100 (1% w/v). When the eluate gave a zero reading of protein, a 0-400 mmol/l gradient of NaCl in the same buffer was applied, followed by the competitive inhibitor, edrophonium chloride (10 mmol/l in buffer) and finally NaCl (1 mol/l in buffer). The elution profile is shown in Fig.III.1. The enzyme eluted in the edrophonium peak only gave a purification of 5-fold and contained 31% of the total AChE and 6% of the protein applied to the column.(Fig.III.1).

When the NaCl gradient was omitted two peaks of AChE correspondingly disappeared but there was a concommitant increase in the AChE activity eluted in the 1 mol/l NaCl peak. There was no effect observed on the total enzyme activity in the edrophonium chloride peak and also very

Fig.III.1

Elution Profile of AChE from MAP-Agarose Column. NaCl Gradient was included.

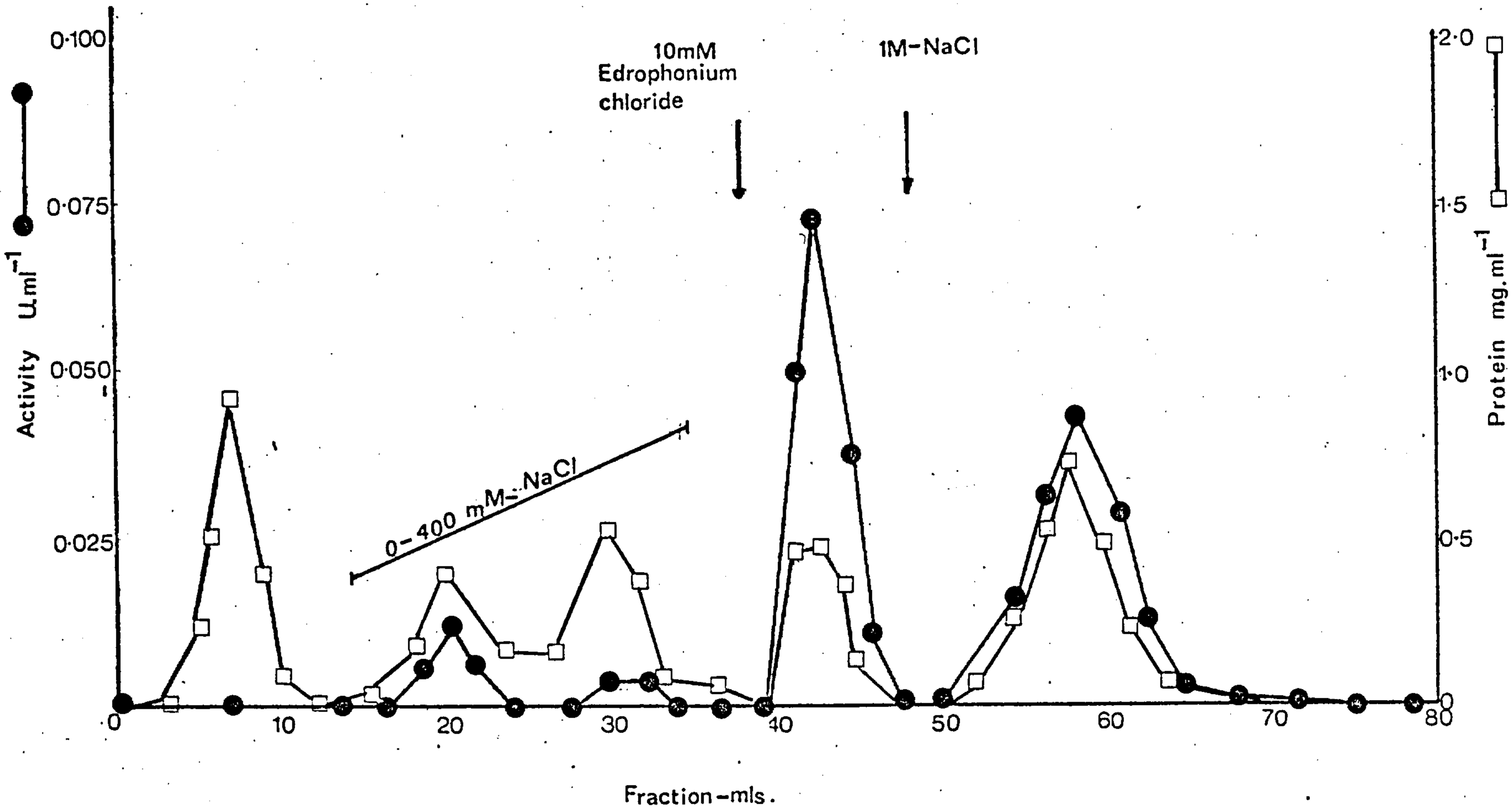


Fig. III. 2

Elution Profile of AChE from MAP-Agarose Column

NaCl Gradient was excluded

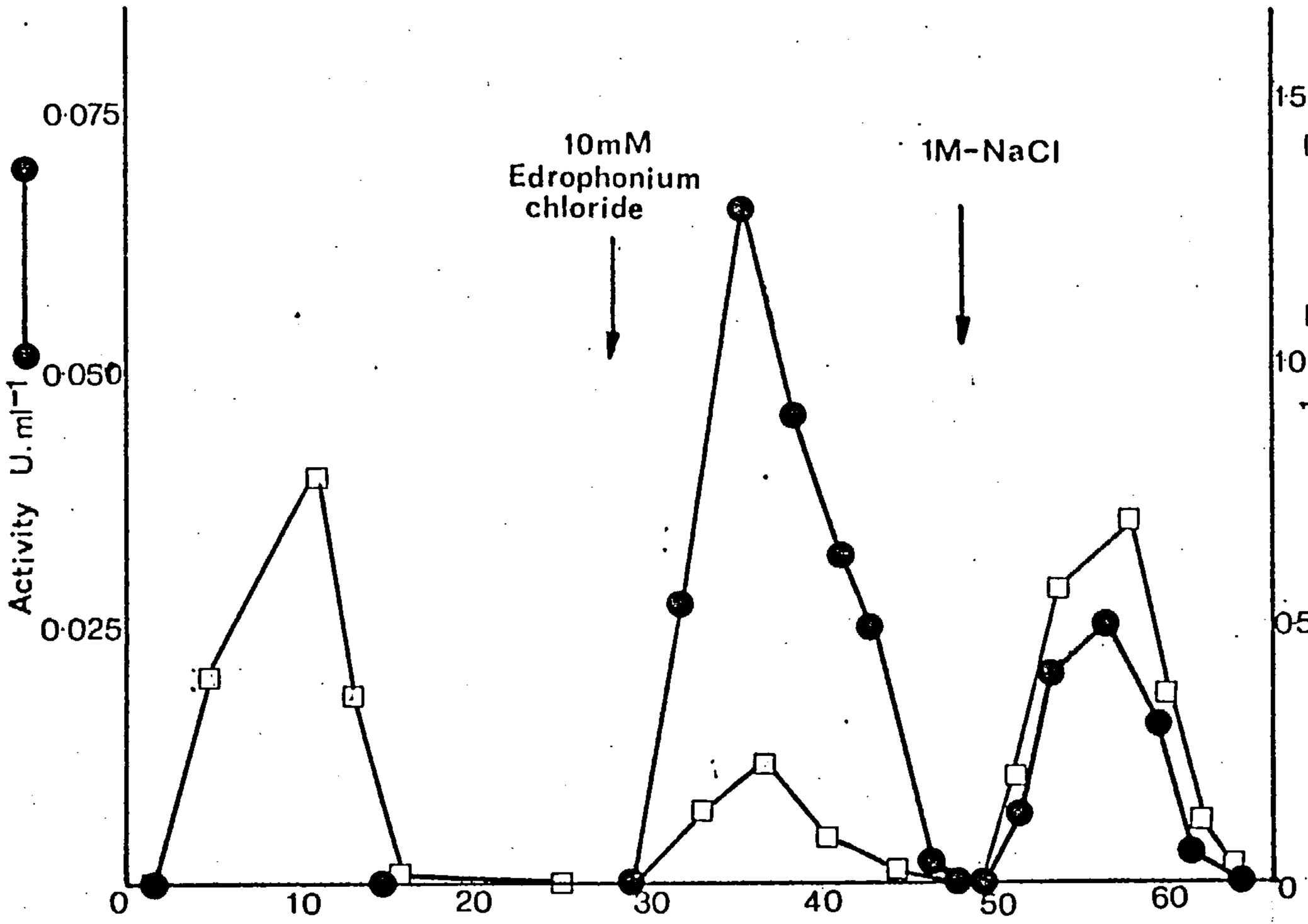


Table III. 7

Yields and Purifications of AChE from MAP-Agarose columns

Elution	Yield from edrophonium Cl peak	Purification from edrophonium Cl peak
Fig. III.1	31%	5
Fig. III.2	35%	6

little difference in the protein eluted in this peak, (Fig.III.2)

When Triton X-100 was excluded from the elution media with the intention of obtaining a detergent free enzyme, the AChE was quantitatively adsorped on to the column and neither 10 mmol/l edrophonium chloride nor 1 mol/l NaCl would remove it until Triton X-100 was reintroduced into the buffer.

Table III.7 compares the two elution protocols shown in Figs III.1 and III.2, and the effect on purification.

(ii) ∈ -Amino caproyl-PTA-agarose column A greater volume of 'Triton solubilized' AChE than was used for the MAP column was passed through the ∈-amino caproyl-PTA column in the hope that more enzyme would be adsorped. For the initial elutions, the column was first equilibriated with sodium phosphate buffer (0·03 mol/l. pH 7·0) containing 1% w/v Triton X-100. When all the free ligand had been washed off, between 100-150 ml. of the crude enzyme was applied to the column at a rate of 30 ml/h. The column was then washed with the buffered Triton X-100 until protein could no longer be detected in the eluate. At this stage the buffer was changed to sodium phosphate buffer (0·03 mol/l. pH 7·0) containing Triton X-100 (1% w/v) and edrophonium chloride (10 mmol/l). When the elution of protein was complete the column was subjected to a final elution of buffer containing NaCl (1 mol/l). The elution profile is shown in Fig.III.3. Rather surprisingly, the peak of protein corresponding to the edrophonium chloride wash showed very little AChE activity. Only 0·4% of the AChE activity occurred in this peak compared with 22% in the 1 mol/l NaCl peak and there was no significant purification. Only 60% of the total protein was eluted from the column indicating that there was still protein adsorped to the agarose. Even when the concentration of edrophonium chloride was increased 5-fold to 50 mmol/l there was little effect on the yield or purification of enzyme.

Fig. III.3

Elution Profile of AChE from ϵ-Aminocaproyl-PTA-Column using Edrophonium Chloride

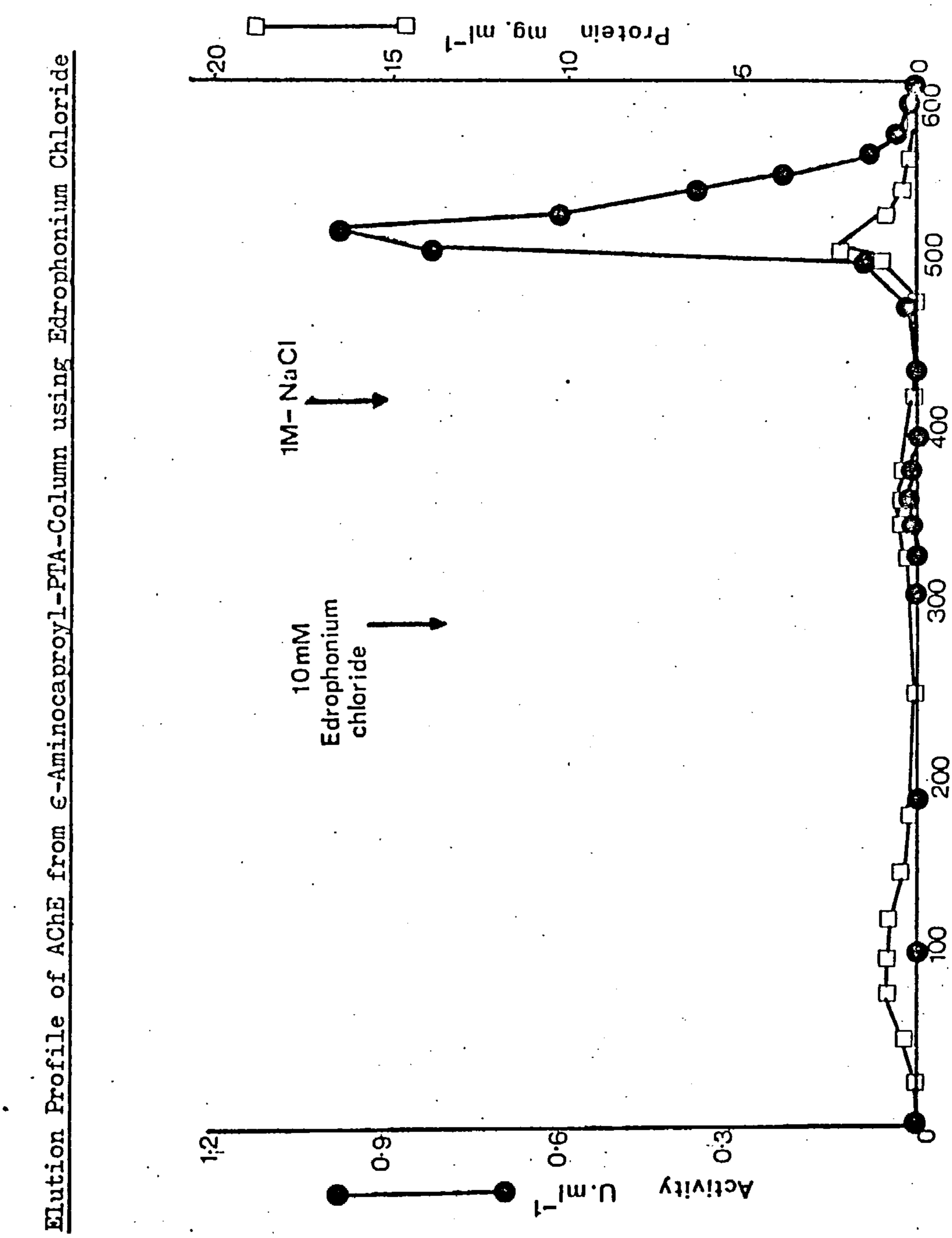
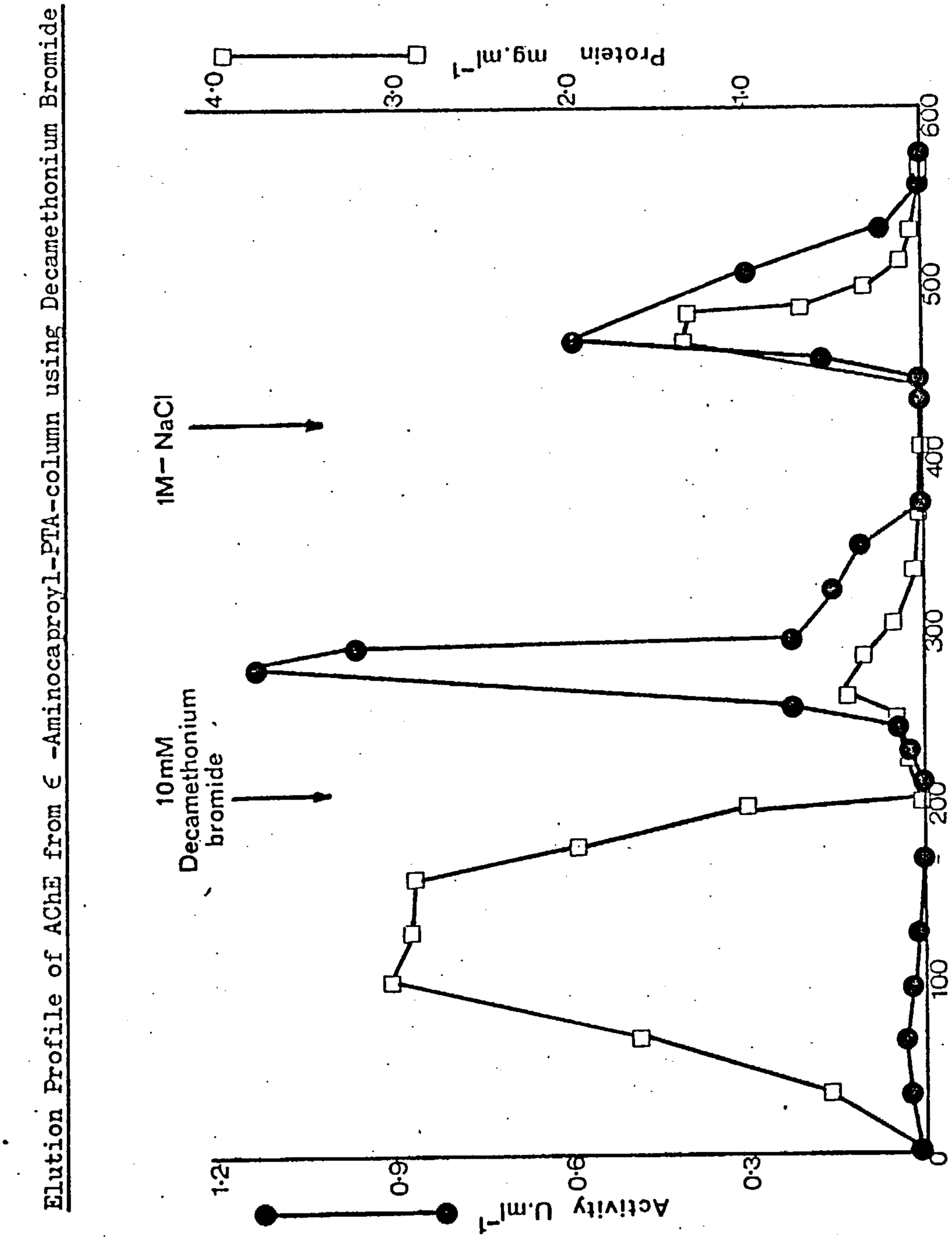


Fig. III.4 Elution Profile of AChE from ∈ -Aminocaproyl-PTA-column using Decamethonium Bromide



A different inhibitor was therefore used to remove the AChE from the column. The inhibitor selected was decamethonium bromide. The elution protocol was the same as previously described except that decamethonium bromide (10 mmol/l) was substituted for edrophonium chloride (Fig.III.4). There was a significant improvment in purification when this modification was introduced. The decamethonium bromide peak yielded 35% of the enzyme activity applied to the column and after the inhibitor had been removed by dialysis the AChE showed a 16-fold purification. However, 40% of the protein still remained adsorped to the column accounting for approximately 50% of the AChE which had been applied to the column. The only way in which the remaining protein could be removed was to wash the column with guanidine hydrochloride (6 mol/l) which denatured the enzyme irreversibly.

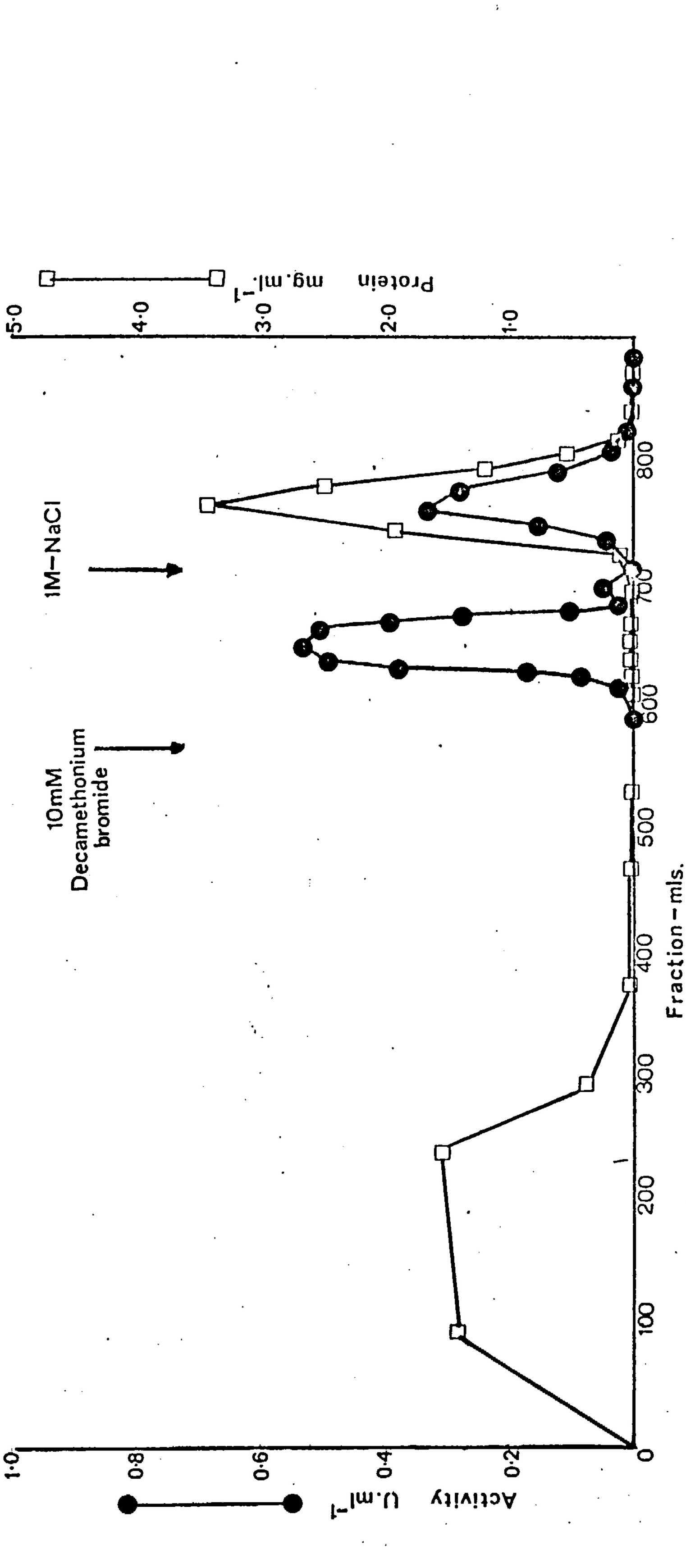
When Triton X-100 was excluded from the elution buffer the enzyme could not be removed even by edrophonium chloride (50 mmol/l), decamethonium bromide (50 mmol/l) or NaCl (2 mol/l).

(iii) MAC-agarose column. As with the ϵ-aminocaproyl PTA-Sepharose, large volumes of the crude detergent solubilized AChE could be applied to the MAC-column without any of the enzyme passing through the column. The agarose was equilibriated with sodium phosphate buffer (0·03 mol/l;pH 7·0) containing Triton X-100 (1%) until all the soluble MAC was removed. The crude enzyme was applied and eluted with the buffered detergent followed by the same medium containing decamethonium bromide (10 mmol/l) and finally with the phosphate buffer containing NaCl (1 mol/l). Between each stage of elution, the level of protein was allowed to fall to zero before continuing with the next stage. (Fig.III.5).

This affinity column gave by far the best results both for purification and for yield of AChE. A total of 44% of the enzyme activity applied was eluted from the column by decamethonium bromide which showed after dialysis a purification of 901 and a specific activity of 148 $\mu mol.min^{-1}.\ mg^{-1}$. When this purified enzyme was rechromatographed on another MAC-column and

Fig.III.5

Elution Profile of AChE from MAC-column Using Decamethonium Bromide



eluted with decamethonium bromide, the specific activity was at least doubled although the protein concentration could not be determined accurately due to its low level.

2. Multiple Molecular Forms

A. Sucrose Density Gradient Centrifugation

When three standard proteins were run on density gradients there was found to be a direct relationship between the sedimentation coefficients and distance migrated down the tube for both the large and small sucrose gradients (Fig.III.6) This relationship was used in future centrifugations by comparing the migration distances of catalase and AChE.

When fresh preparations of 'Triton solubilized' AChE were centrifuged on sucrose gradients, only one peak of enzyme activity was resolved corresponding to 11-12S or a M.W. of 240,000 to 250,000 (Fig.III.7). When 5 day old preparations of 'Triton solubilized' AChE were centrifuged the basal level of enzyme activity throughout the gradient tended to increase and could very rarely be resolved into individual peaks of approximately 20S and 25S (MW's 570,000 and 780,000) although results were very variable. However, the 11-12S species was always present in the gradient. When a high salt concentration (1 mol/l NaCl) was incorporated into the sucrose, there was a tightening up of the 11-12S peak (Fig.III.8)

The 'soluble' AChE consistently showed peaks of activity at 11-12S with shoulders at 15S and 19S (Fig.III.9). These shoulders corresponded to MW's of 360,000 and 525,000 respectively. No molecular species of less than 11-12S were ever observed.

The 'EDTA solubilized' AChE also showed several peaks of activity corresponding to different multiple molecular forms (Fig.III.10). Again the 11-12S species predominated with shoulders at 14·5S and 19S.

When the 11-12S peaks from any preparation were re-run on the sucrose gradients, the sedimentation coefficients were found to remain at 11-12S. However, when the 15S or 19S peaks were recentrifuged, the new profiles could not be resolved but there was a broad plateau of activity in the

Fig. III.6

Sedimentation Behaviour of Muramidase, Alcohol Dehydrogenase and Catalase on 25ml. Gradients & 5ml. Gradients

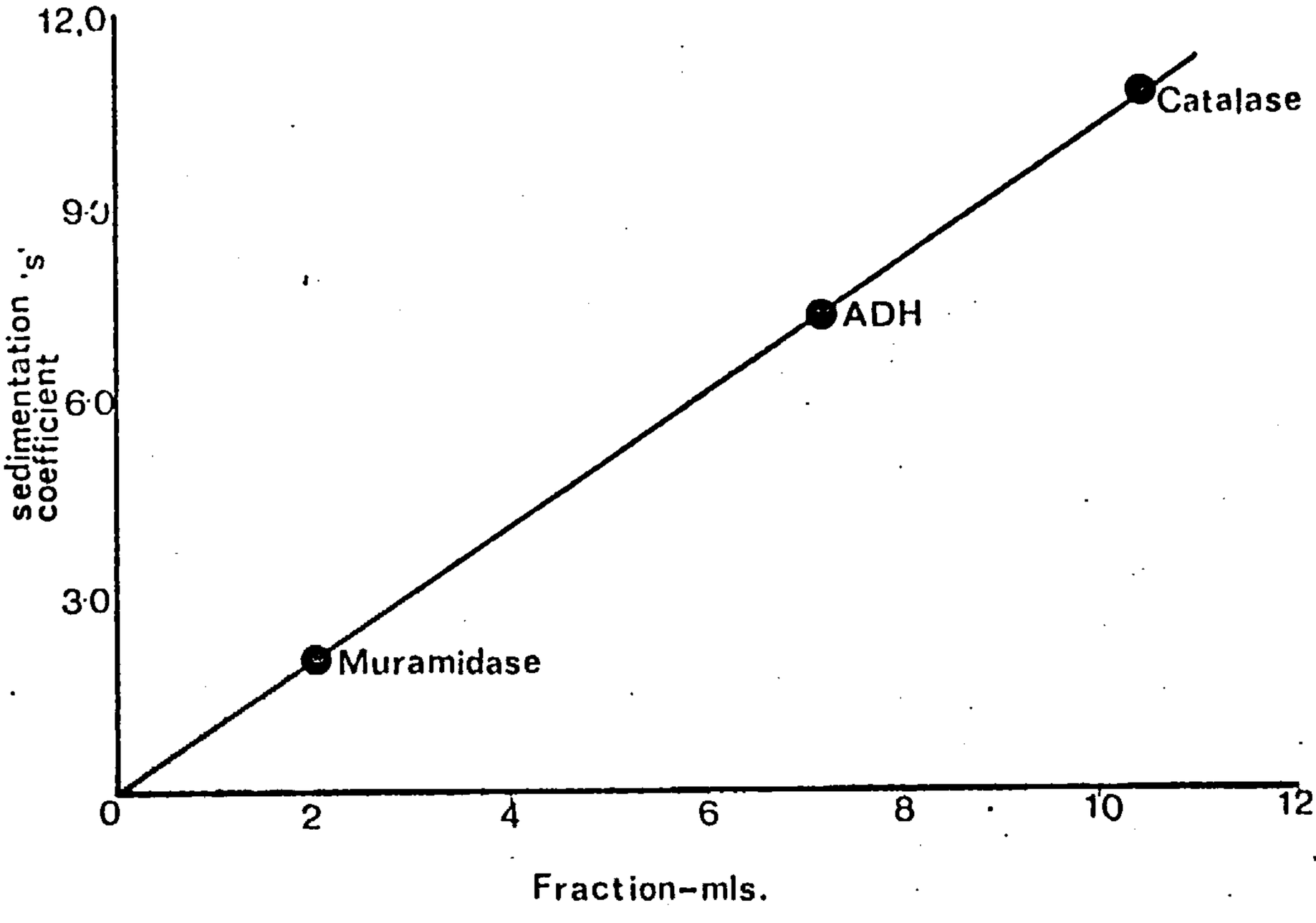


Fig. III.7

Sucrose Density Gradient Centrifugation of Triton X-100 Soluble AChE

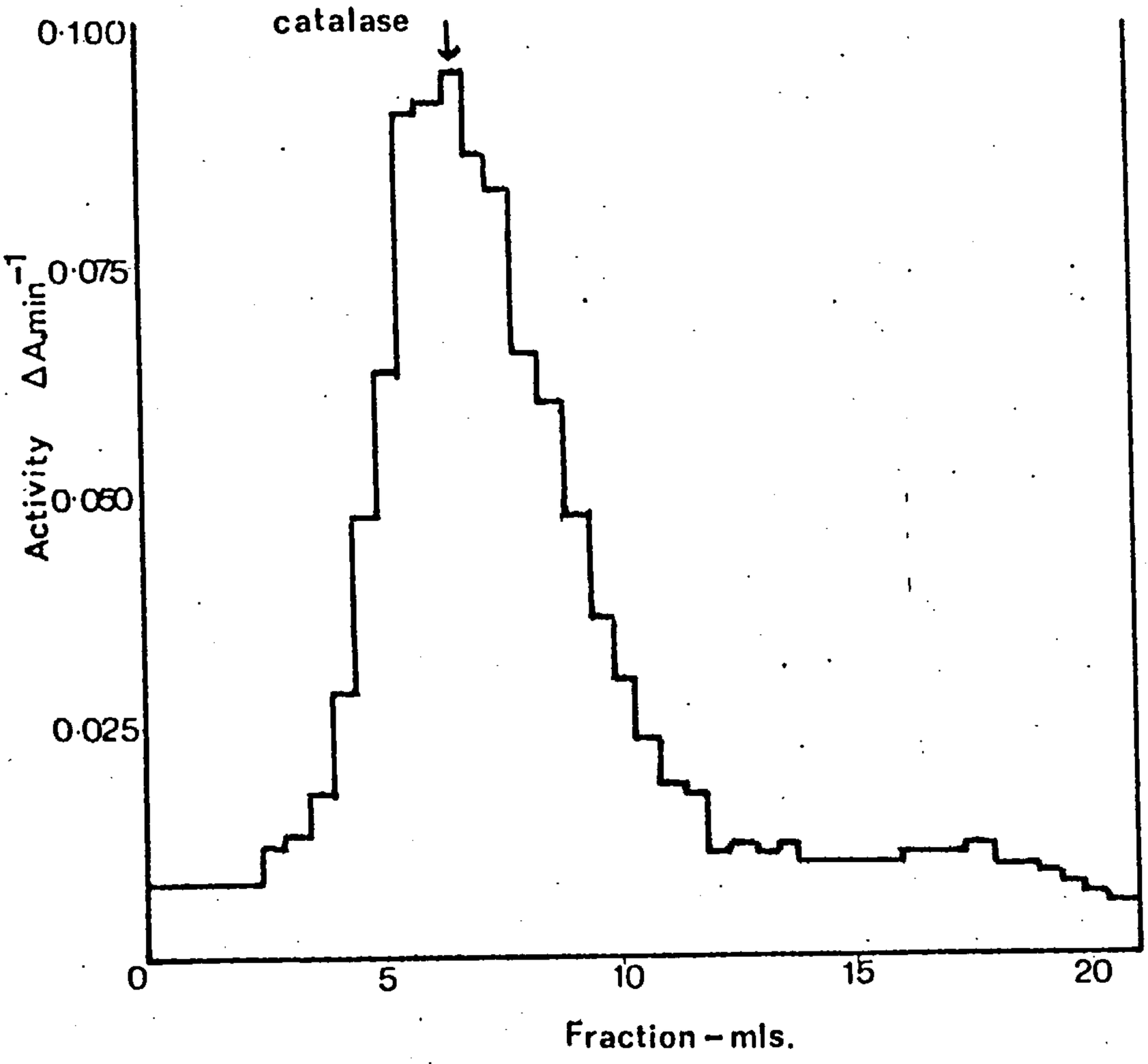


Fig.III.8

Sucrose Density Gradient Centrifugation of 'Triton soluble AChE' Incorporating 1 mol/l NaCl

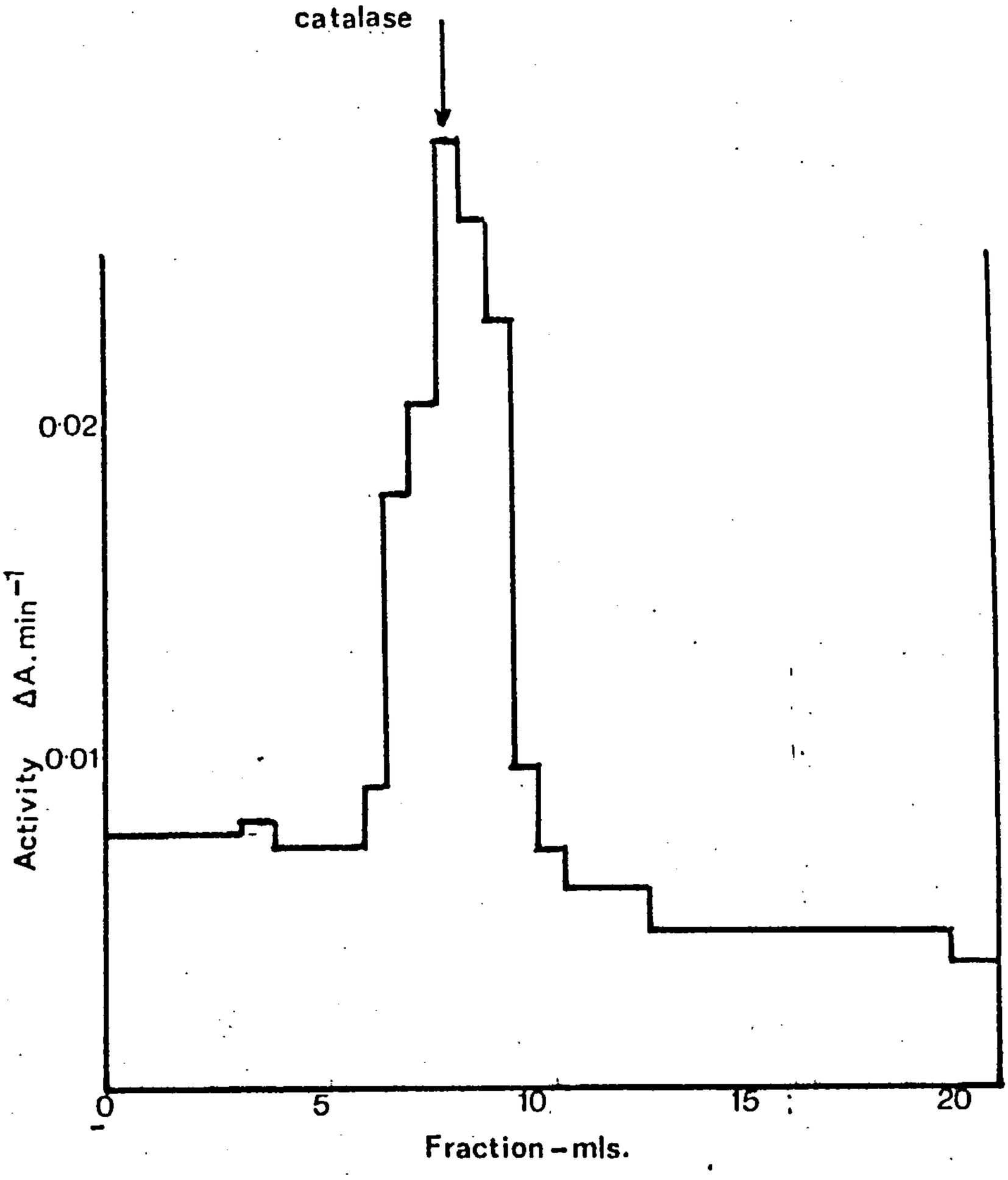


Fig.III.9

Sucrose Density Gradient Centrifugation of Soluble AChE

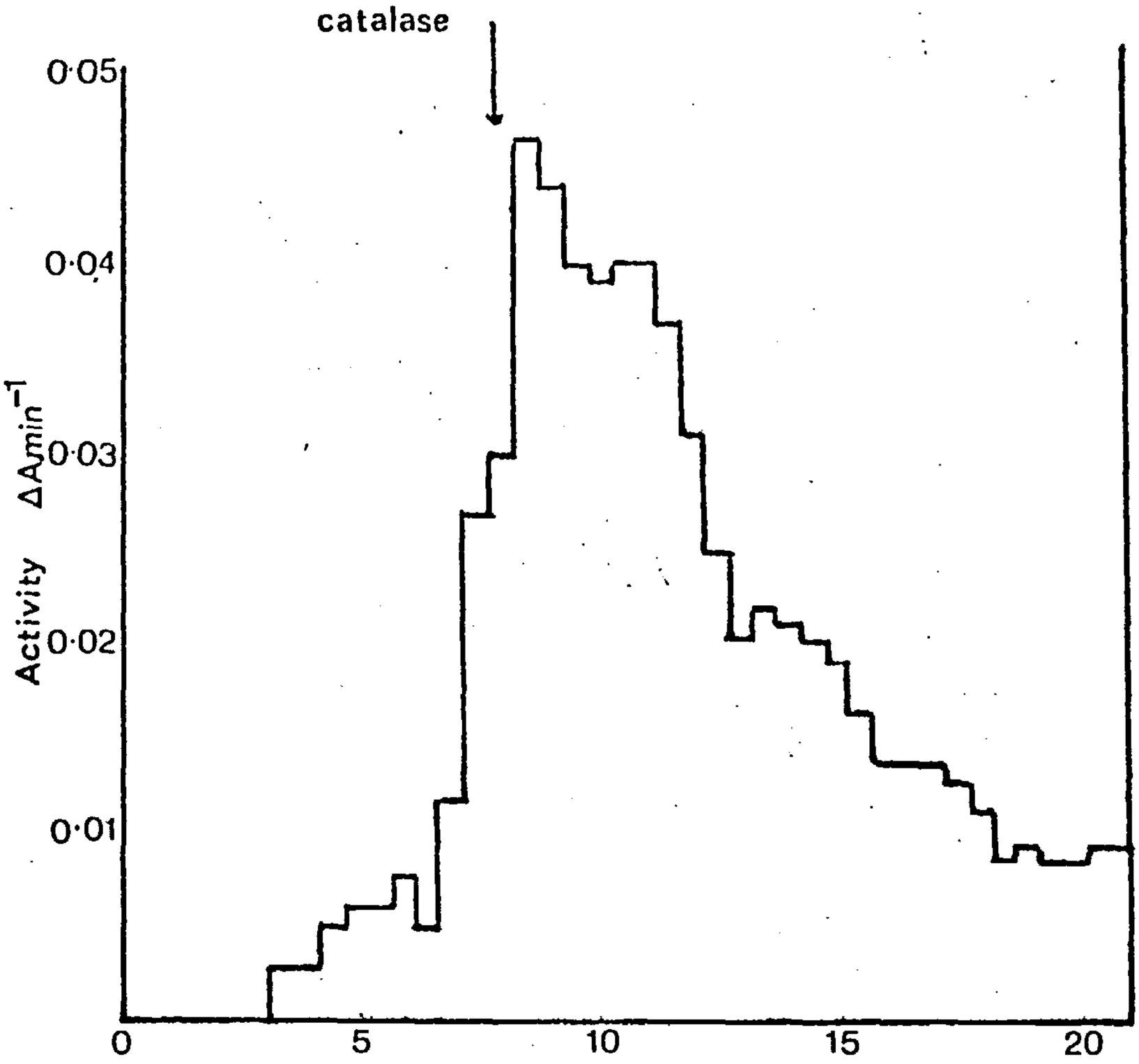
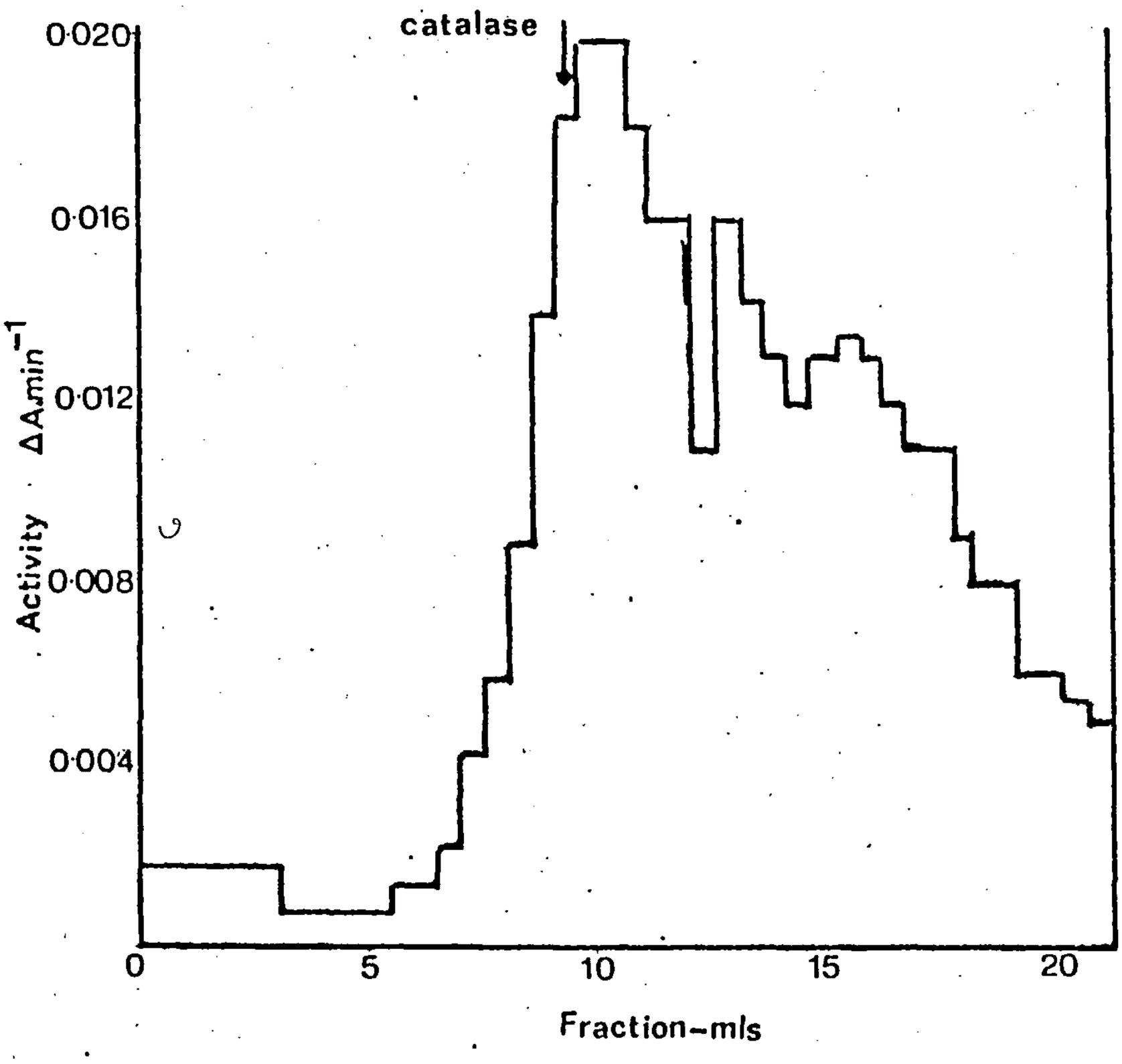


Fig.III 10

Sucrose Density Gradient Centrifugation of EDTA solubilized AChE



10S-20S range.

The peaks of 'Triton solubilized' and 'soluble' AChE resolved by starch block electrophoresis were also subjected to density gradient centrifugation. In the case of 'Triton solubilized' AChE, when the one peak present on the starch block containing no detergent was centrifuged, it migrated quickly to the bottom of the gradient suggesting a very high molecular weight species. The same thing happened to peak I from the starch block containing Triton X-100. However, the peak II from this starch block gave a value of 11-12S on the sucrose density gradient.

In the case of the 'soluble'AChE, peak I of activity migrated to the bottom of the tube and peak II showed a sedimentation constant of 11-12S.

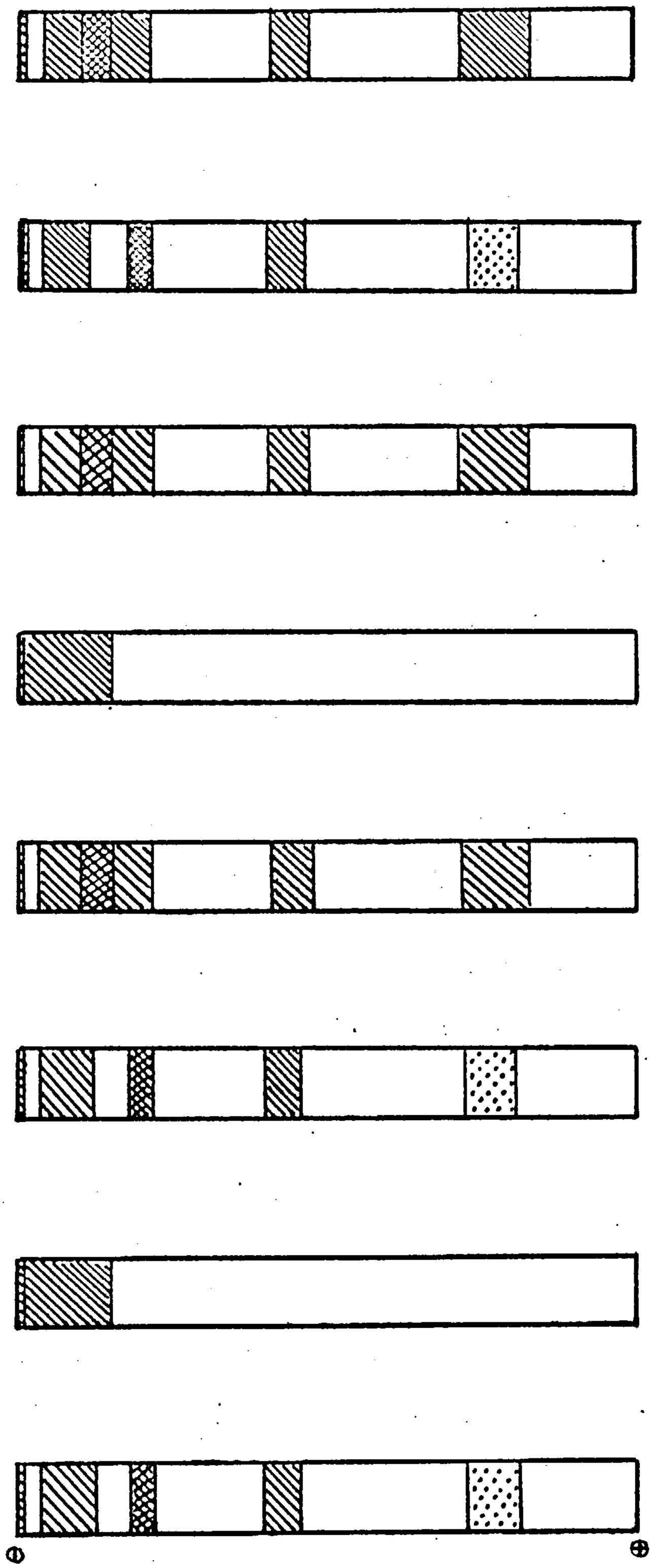
AChE purified by affinity chromatography was also centrifuged on a sucrose gradient and was found to have the same sedimentation characteristics as the crude 'Triton solubilized' enzyme both before and after starch block electrophoresis.

B. Polyacrylamide Gel Electrophoresis

i. Polyacrylamide rods. The enzyme band patterns are shown in Fig.III.11. The 'Triton solubilized' enzyme always gave three characteristic bands of activity, the fastest moving band however being quite faint. If Triton was not incorporated in the gel with the detergent solubilized enzyme, very dense and diffuse staining was observed at the cathodic end. This phenomenon occurred whenever there was Triton in the enzyme preparation but absent from the gel. Peaks I and II from the starch block electrophoresis of 'Triton soluble' or 'soluble'AChE were also electrophoresed on polyacrylamide rods. The peak I from both preparations only just entered the gels causing diffuse staining at the cathodic end while peak II gave a similar staining pattern as the crude 'detergent solubilized' or 'soluble' enzyme. When enzyme preparations were left for 5 days before electrophoresis, enzyme staining became more intense near the cathodic end of the rods and fainter at the anodic end.

Fig.III.11

Polyacrylamide Gel Rod Electrophoresis of AChE (2·5mA per tube, 1½h)



The AChE inhibitor BW 284 C51 dibromide completely abolished all enzyme staining. When butyryl thiocholine iodide was used as substrate in the stain, very faint bands of activity appeared but did not correspond to those produced by AChE.

The staining pattern for affinity purified AChE resembled very closely that of the crude 'Triton solubilized' enzyme, but the bands of activity were much sharper. Also, the peaks I and II of the starch block electrophoresed purified enzyme gave the same pattern as the crude 'Triton solubilized' enzyme in polyacrylamide rods.

ii. Polyacrylamide slabs. Electrophoresis in polyacrylamide gradients also showed a multiplicity of bands (Fig.III.12). Assuming that the AChE molecules are spherical, then the molecular weights obtained for the 'soluble' enzyme were 340,000, 260,000, 135,000, and 68,000. The 'Triton solubilized' AChE had three bands in common with the 'soluble' enzyme with molecular weights at 365,000, 264,000 and 68,000. In addition there was a 181,000 species and a faint band at 83,000. Peak II from the starch block electrophoresis of 'Triton solubilized' or 'soluble' AChE gave the same patterns as before electrophoresis on starch blocks but the peak I showed a very high molecular weight entity only just entering the gel.

The EDTA extracted enzyme gave a band at 250,000 and also others at 160,000, 124,000 and 84,000. The one feature in common with the various preparations is a M.W. species in the region of 250,000 which was also the major species found after sucrose gradient centrifugation.

The staining pattern for electrophoresis of the affinity chromatography purified enzyme was very different from all other patterns. As well as bands at 240,000, 68,000 and 185,000, there were bands at 110,000 and 125,000. It was found that the multiple molecular forms were resolved into much sharper bands if Triton X-100 was incorporated into the gel.

C. Starch Block Electrophoresis

When the 'Triton solubilized' enzyme was electrophoresed on a starch

Fig.III.12

Polyacrylamide Slab Gel Electrophoresis of AChE (100v., 24h)

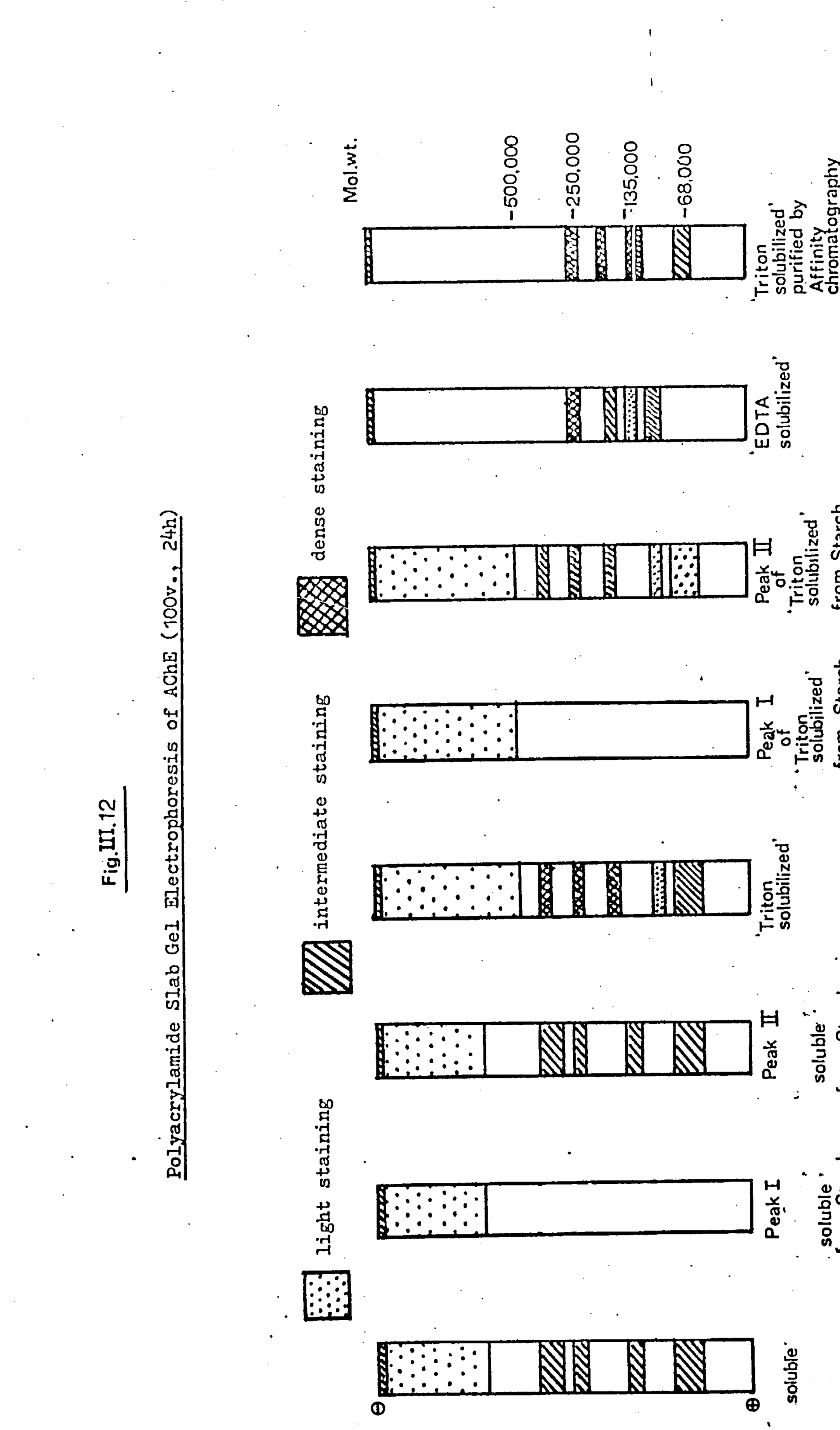


Fig. III. 13

Starch Block Electrophoresis of 'Triton Solubilized' AChE.

(Detergent excluded from Block)

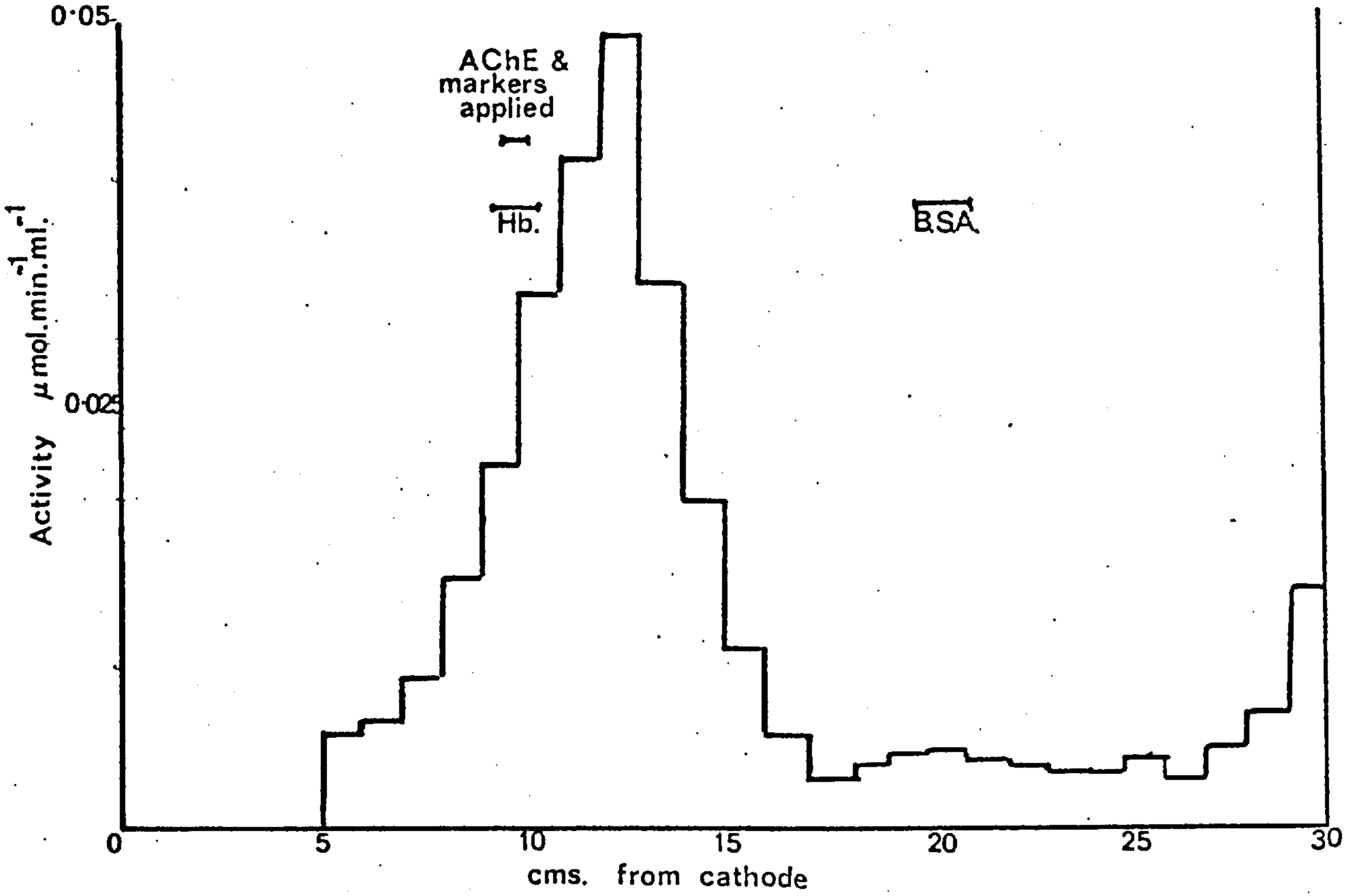


Fig. III. 14

Starch Block Electrophoresis of 'Triton Solubilized' AChE

(1% w/v Detergent included in Block)

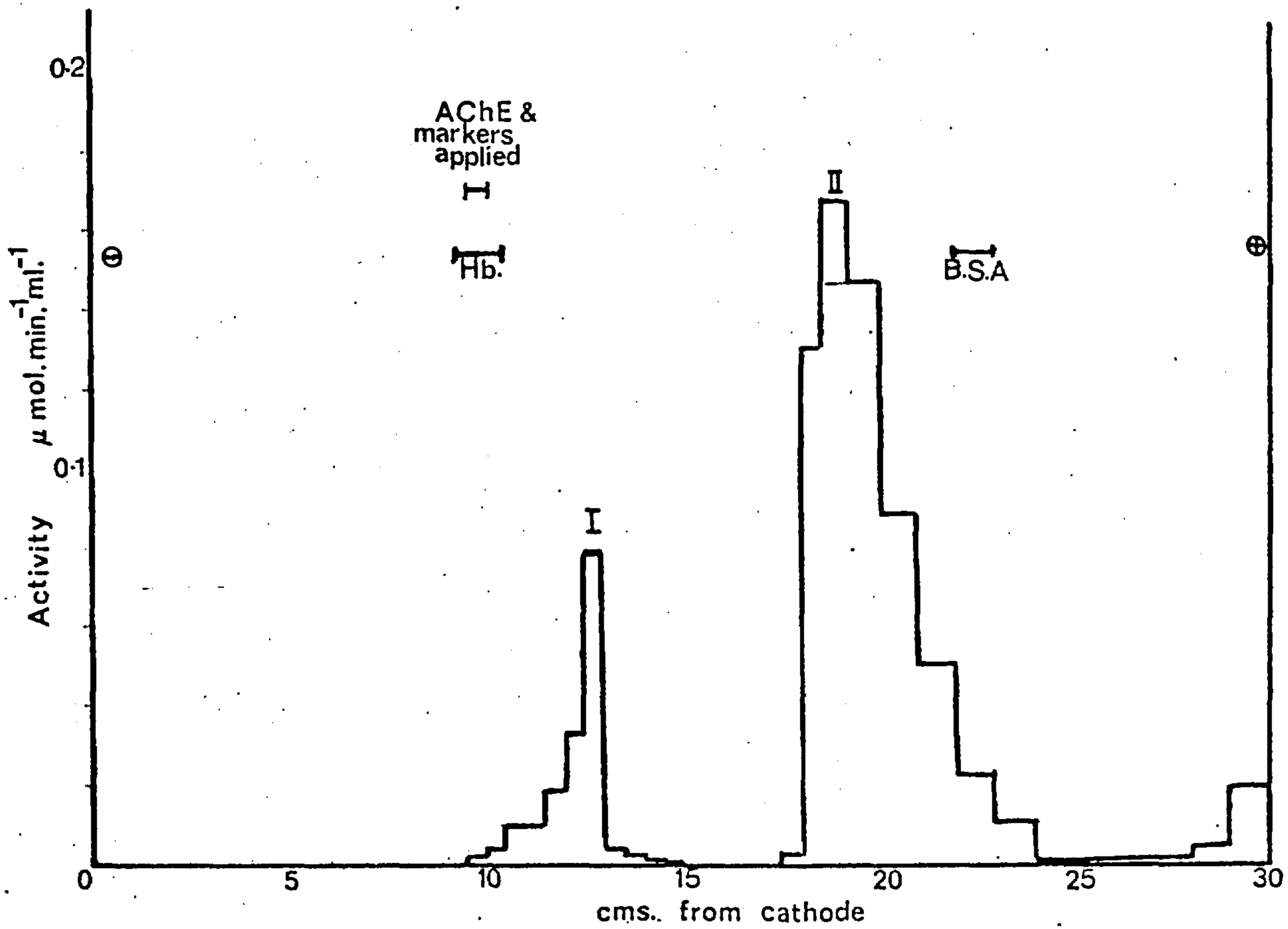
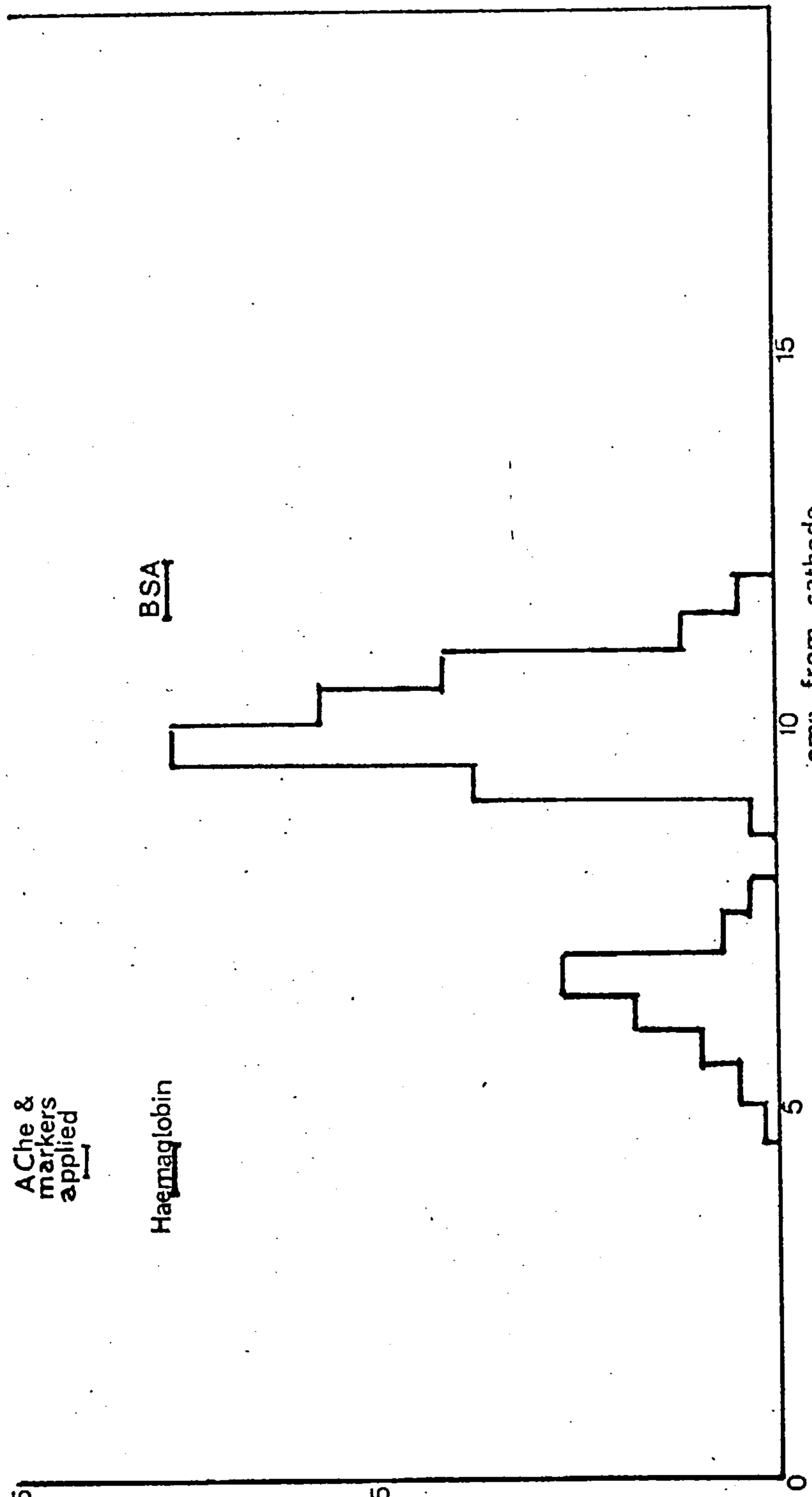


Fig III. 15

Starch Block Electrophoresis of 'Soluble' AChE



block with the detergent excluded from the system, one major peak of activity was resolved which was quite slow moving (Fig.III.13). By incorporating Triton X-100 into the system before electrophoresis the enzyme could be resolved into two separate entities; a slow moving peak (I) and a fast moving peak (II) (Fig.III.14). Sometimes a very small additional peak appeared in the fractions adjacent to the anode. When peaks I and II were electrophoresed they maintained their integrity as individual entities. The 'soluble' AChE also showed two peaks of activity (Fig.III.15) which remained as separate entities when re-electrophoresed on the starch block. The affinity chromatography purified enzyme showed exactly the same characteristics as the crude detergent solubilized AChE when subjected to starch block electrophoresis.

D. Effect of Removing Triton X-100 from the Preparation AChE

When 'Triton solubilized' AChE was passed through a hydroxylapatite column, most of the enzyme was adsorped to the calcium phosphate gel and could not be removed until 0·1% w/v Triton X-100 was reintroduced. The small amount of enzyme which was eluted was found to be in a high molecular weight aggregated form by gel and starch block electrophoresis and also by density gradient centrifugation.

E. Treatment of AChE with DEAE-Sephadex

Treatment of preparations of AChE with DEAE-Sephadex according to Hollunger & Niklasson (1973) failed to dissociate the higher molecular weight forms of the enzyme into lower molecular weight species and had no effect whatsoever on the staining patterns by polyacrylamide gel electrophoresis.

3. Studies on Membrane Bound AChE

Affinity purified AChE was first treated to deplete the Triton X-100 content to0·1% w/v before binding to liposome membranes.

A. Arrhenius Plots of AChE preparations

The pH-stat assay was used to determine the Arrhenius plots of the various preparations of AChE between the temperatures of 5-50°C. The

V_{max} was found from S/v vs S plots calculated on an Olivetti electronic desk top computer. Log V_{max} was then plotted against the reciprocal of the Absolute temperature (Plummer, Reavill, and McIntosh, 1975).

The membrane preparation used was taken as the resuspended 100,000g pellet from the centrifugation of a 20% w/v brain homogenate in 0·03 mol/l sodium phosphate buffer pH 7·0. The Arrhenius plot of the enzyme preparation showed two straight lines with a pronounced break at 27°C, the transition temperature (Fig.III.16). The energies of activation were calculated as 8·3 and 39 kJ/mol. When the enzyme was solubilized by Triton X-100, the break was abolished and the activation energy found to be 20 kJ/mol. (Fig.III.17). Similarly, the 'soluble' form of AChE had no break in its Arrhenius plot, the energy activation being 19 kJ/mol. (Fig.III.18).

B. Effect of Liposomes on Km of purified AChE

(i) Positively charged liposomes(15% stearoylamine,85% phosphatidyl choline)

When the purified AChE was bound to positively charged liposomes, the Km of the enzyme was 150 μM. This compares to a value of 44 μM when the Km was determined in the absence of the liposomes. When Triton X-100 (1% w/v) was added to the liposome-enzyme complex, the Km was lowered towards the value of the free enzyme although it never quite reached the value of 44μM. The V_{max} was not altered significantly by binding to liposomes.

(ii) Negatively charged liposomes (30% myristic acid,70% phosphatidyl choline)

Addition of negatively charged liposomes to purified AChE caused the Km to fall to 29 μM from 44 μM while the V_{max} was unchanged. The addition of Triton X-100 to the liposome-enzyme complex brought the Km back to the region of 44 μM.

C. Effect of Temperature on the Adsorption of AChE to Liposomes

(i) Positively charged liposomes(15% stearoylamine,85% phosphatidyl choline)

When the temperature of the binding was lowered from 25°C. to 4°C. the Kass was changed very slightly from $1.1 \times 10^5 M^{-1}$ to $1.4 \times 10^5 M^{-1}$ as determined from the slopes of the Scatchard plots in Figs.III.19 & 20. The apparent

Fig. III.16

Arrhenius Plot of Membrane AChE

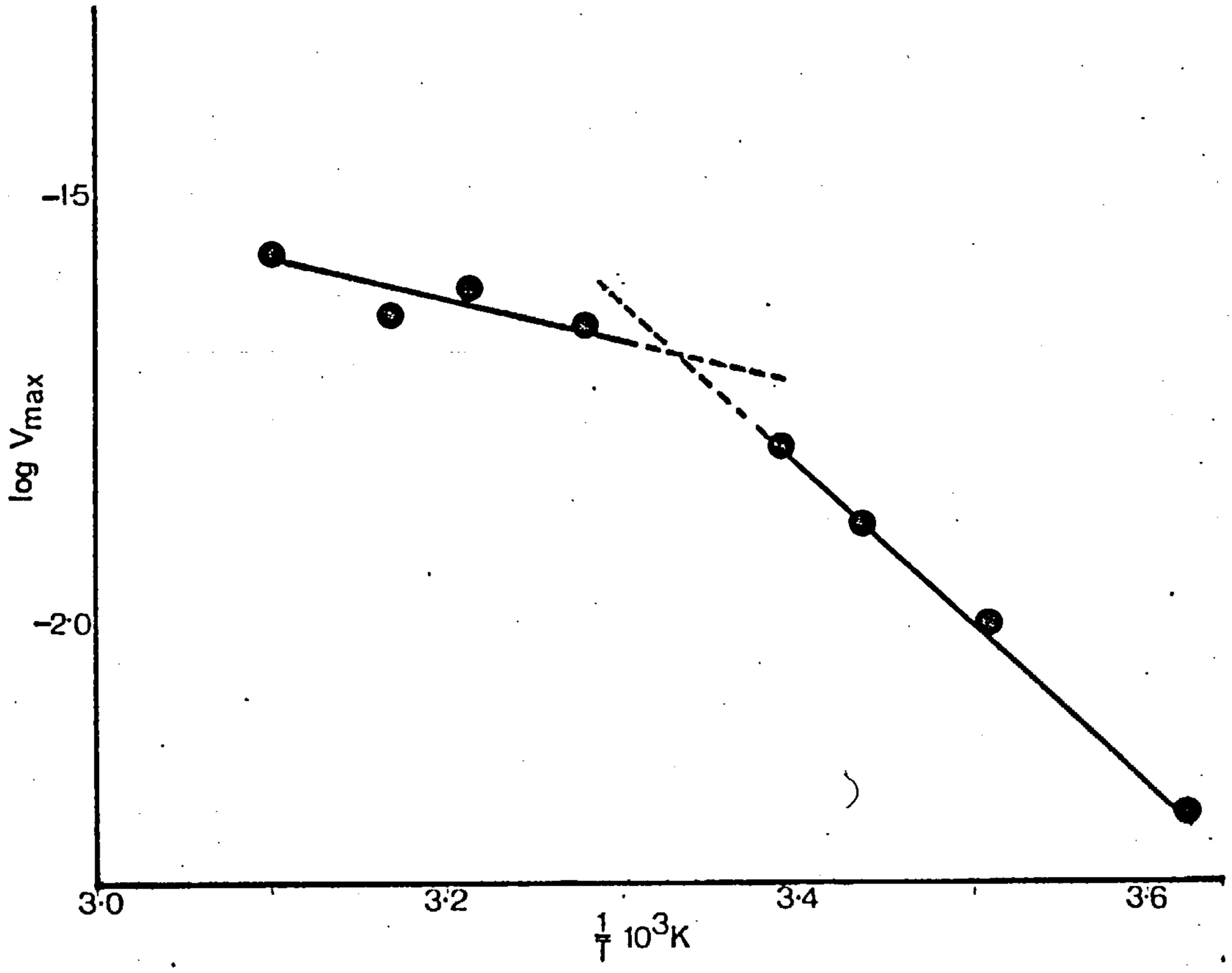


Fig. III.17

Arrhenius Plot of 'Triton Solubilized' AChE

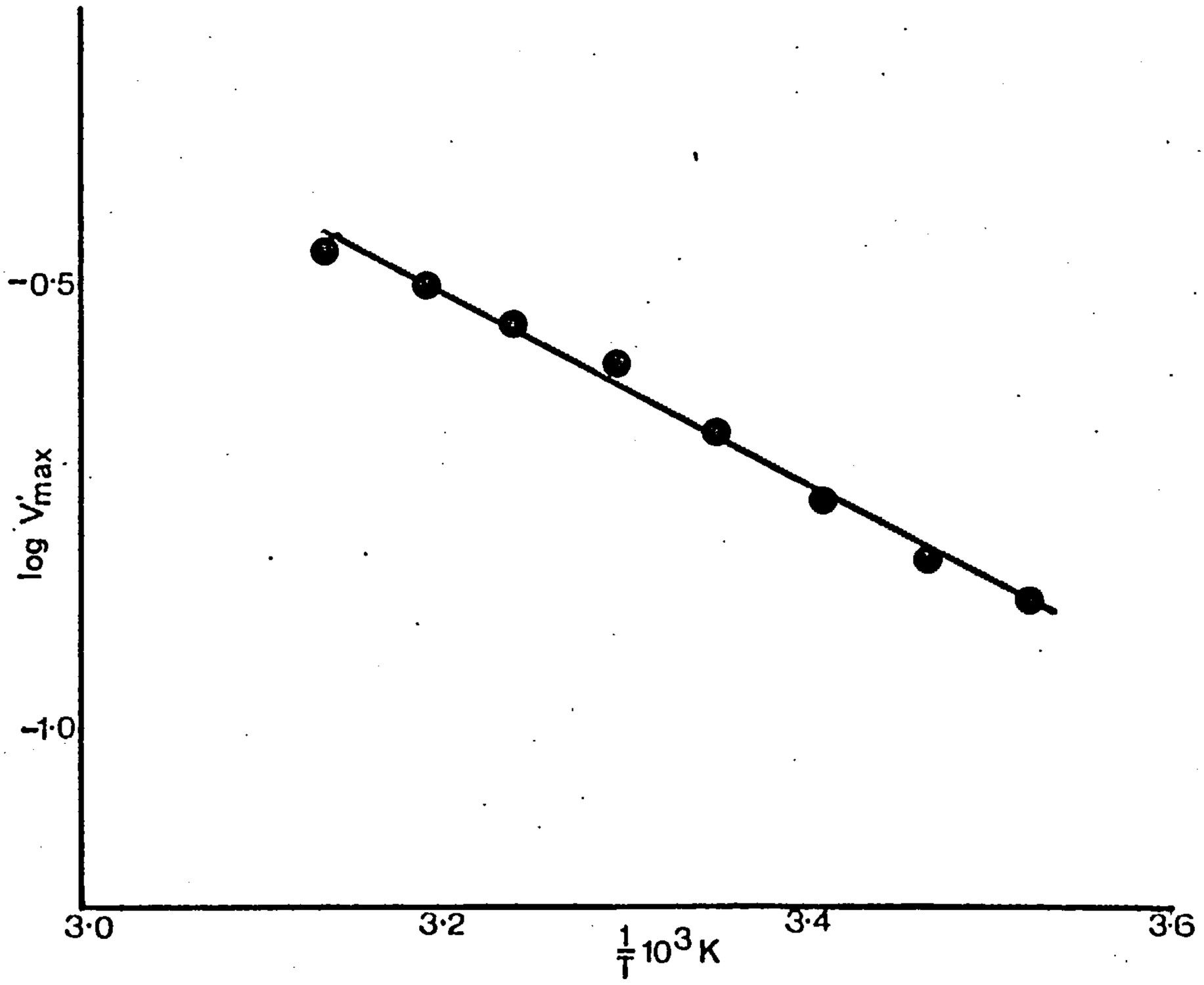
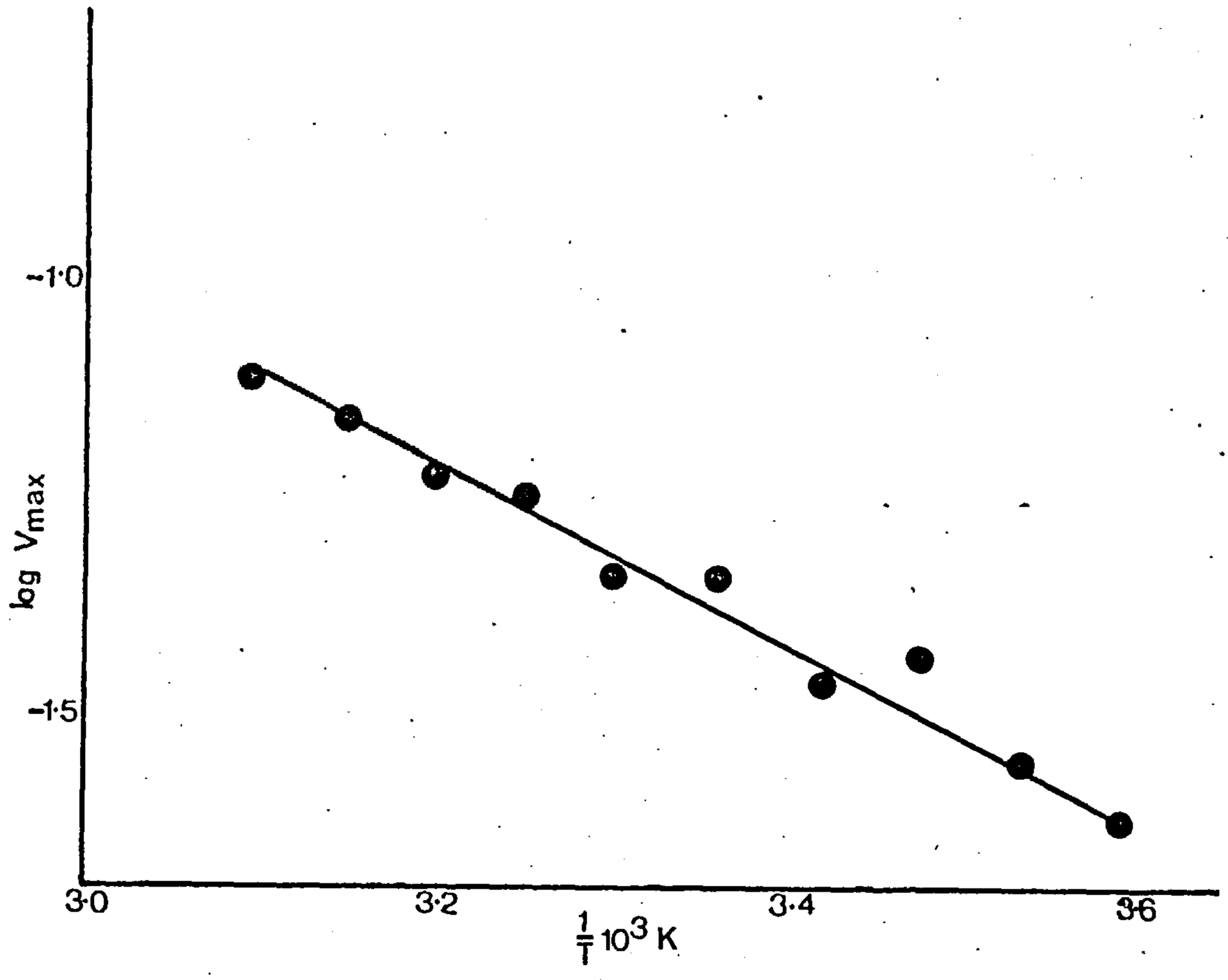
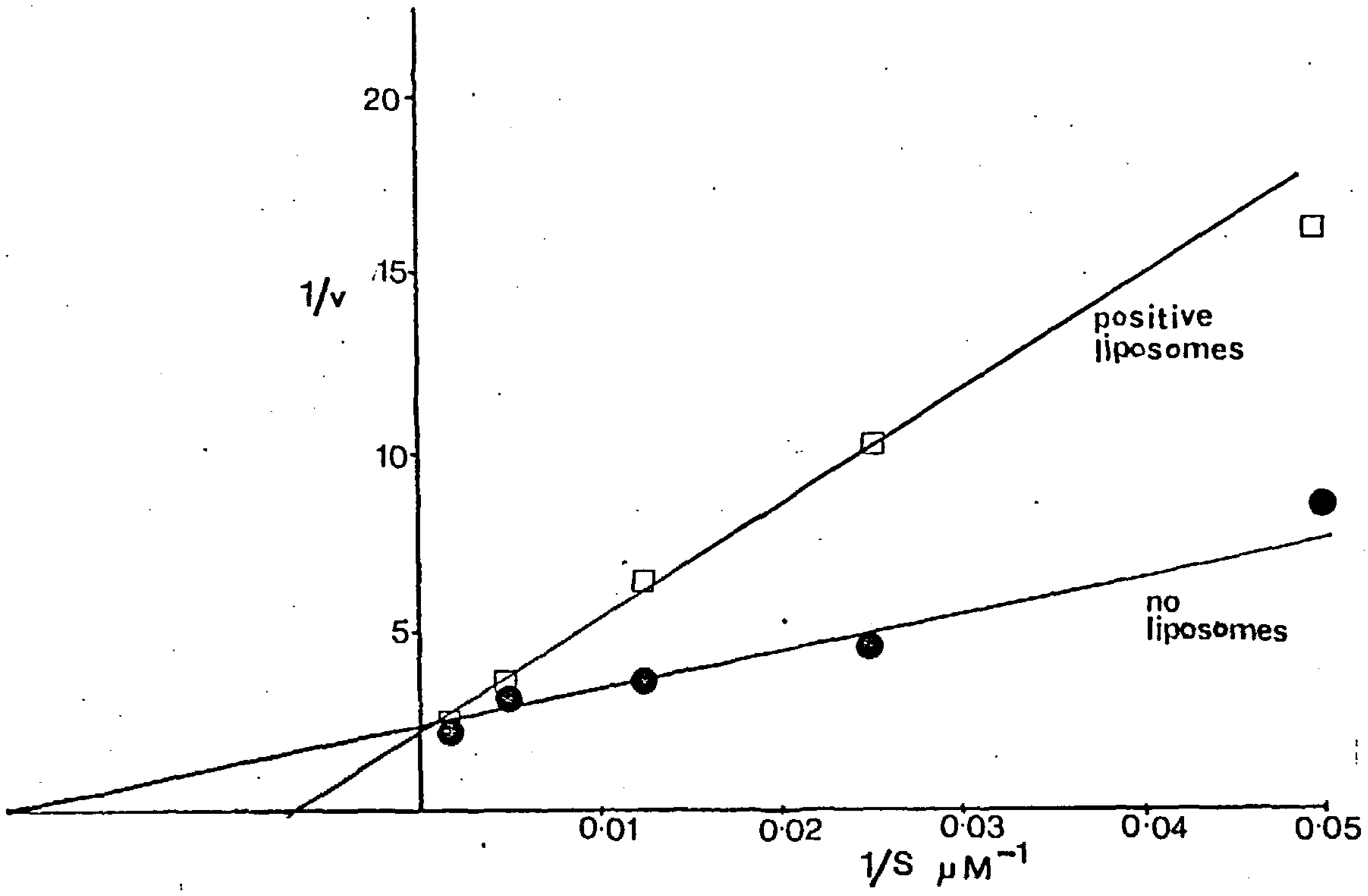


Fig. III.18

Arrhenius Plot of Soluble AChE



Lineweaver-Burke plots for AthCh in the presence □ and absence ● of charged liposomes



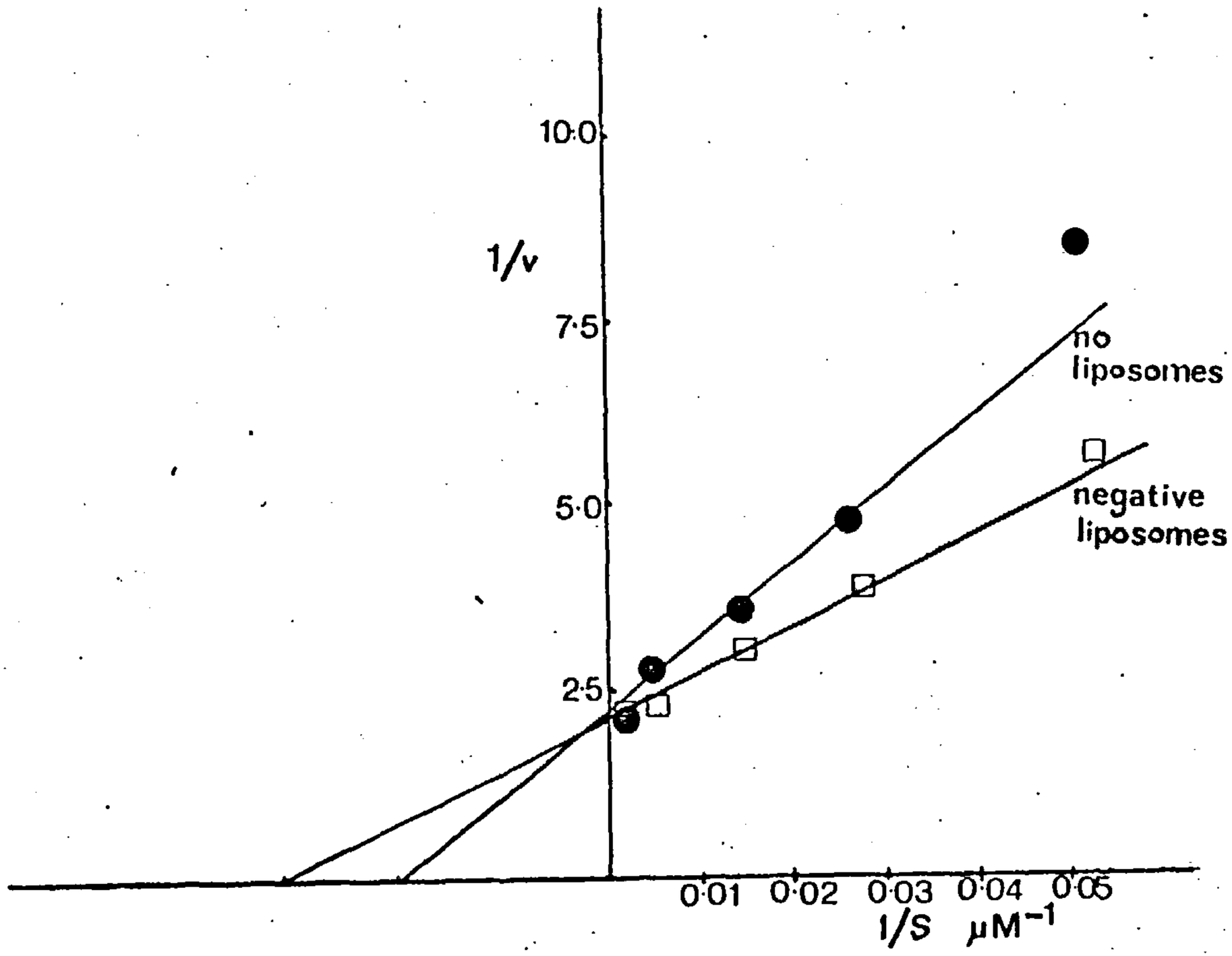


Fig. III. 19

Scatchard Plot of AChE Binding to Positively Charged Liposomes

(15% stearoylamine, 85% phosphatidyl choline)

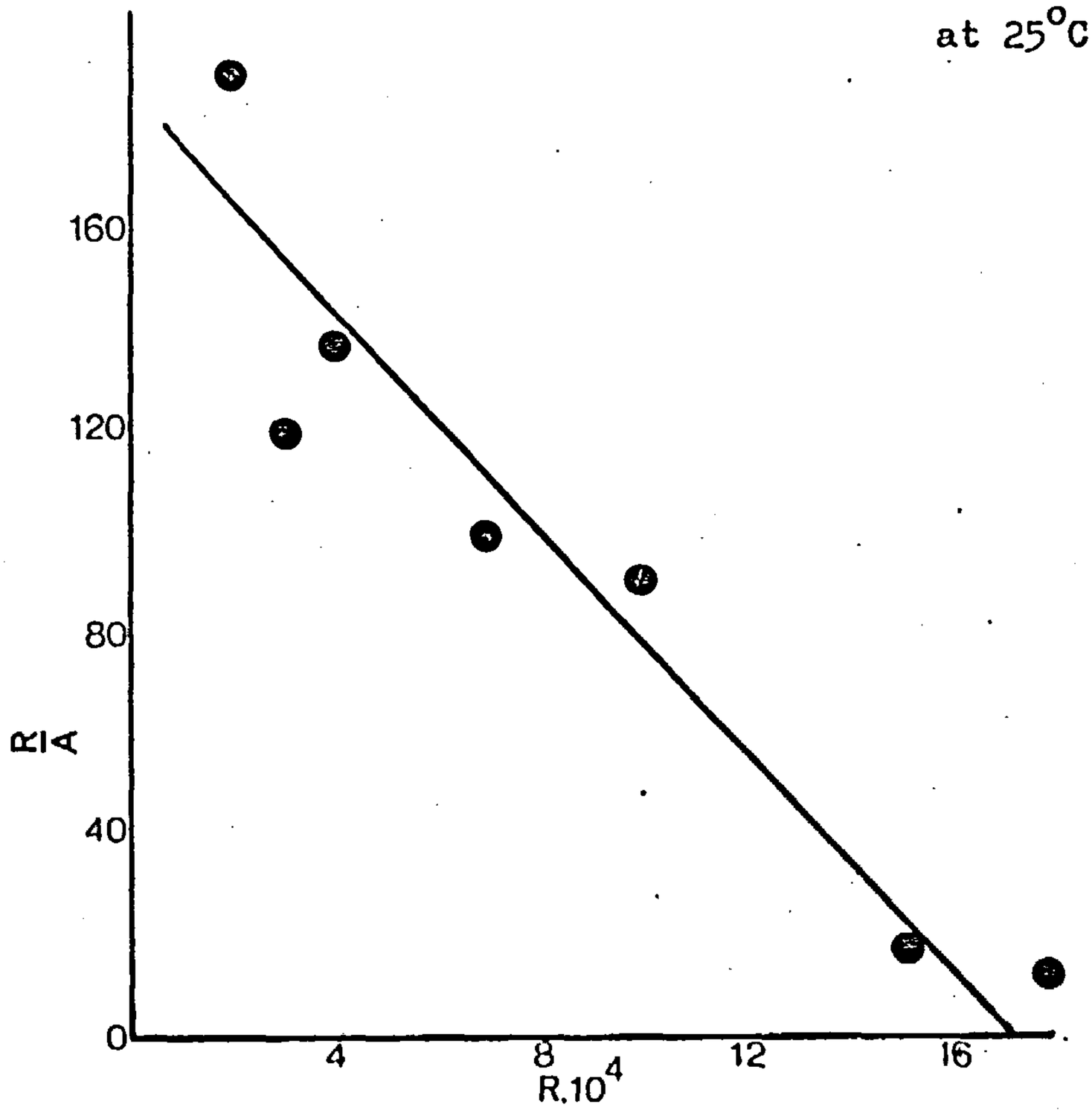
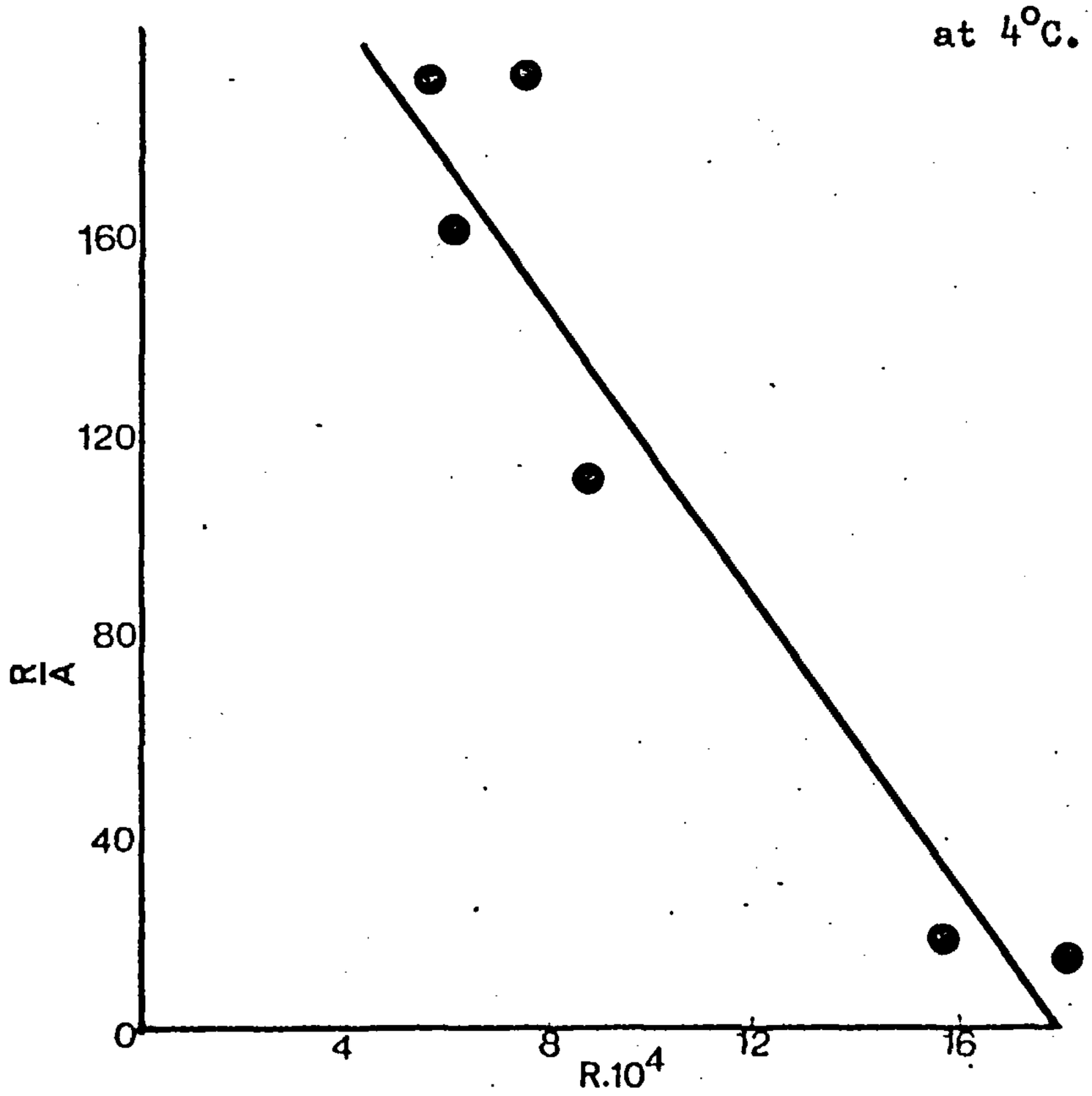


Fig. III. 20

Scatchard Plot of AChE Binding to Positively Charged Liposomes

(15% stearoylamine, 85% phosphatidyl choline)



free energy change ($\Delta G'$) for adsorption found from the equation

$$\Delta G' = -RT \ln Kass$$

changed only slightly from $-27\ kJ.mol^{-1}$ at $4^{o}C.$ to $-29\ kJ.mol^{-1}$ at $25^{o}C.$

The Van't Hoff equation

$$\frac{d(\ln Kass)}{dT} = \frac{\Delta H}{RT^2}$$

was used to determine the enthalpy of the reaction. This was found to be small and negative having the value -8 kJ/mol.

(ii) Negatively charged liposomes(30% myristic acid,70% phosphatidyl choline).

When the AChE was adsorped to negatively charged liposomes, the Kass rose slightly from $3{\cdot}4 \times 10^4\ M^{-1}$ at $25^{o}C.$ to $4{\cdot}0 \times 10^4\ M^{-1}$ at $4^{o}C.$ (Scatchard plots in Figs.III 21 & 22). The $\Delta G'$ showed very little difference, the change being -26 kJ/mol at $25^{o}C.$ to -24 kJ/mol at $4^{o}C.$ The ΔH was again small at -5 kJ/mol.

D. Arrhenius plots of AChE adsorped to Liposomes containing or excluding Cholesterol

The phospholipid, dimyristyl phosphatidyl choline shows a phase change at $24^{o}C.$ (Redwood & Patel, 1974). When AChE was bound to 50% dimyristyl phosphatidyl choline, 50% myristic acid liposomes, the Arrhenius plot showed a phase change at $21^{o}C.$ with activation energies of 7·3 kJ/mol and 14·4 kJ/mol (Fig.III.23). However, when cholesterol (1% mol/mol) was incorporated into the liposomes, the phase change disappeared and the activation energy increased to 20 kJ/mol (Fig.III.23).

E. Effect of AChE adsorption to Liposomes on the Membrane Potential(ψ)

. By using the relationship

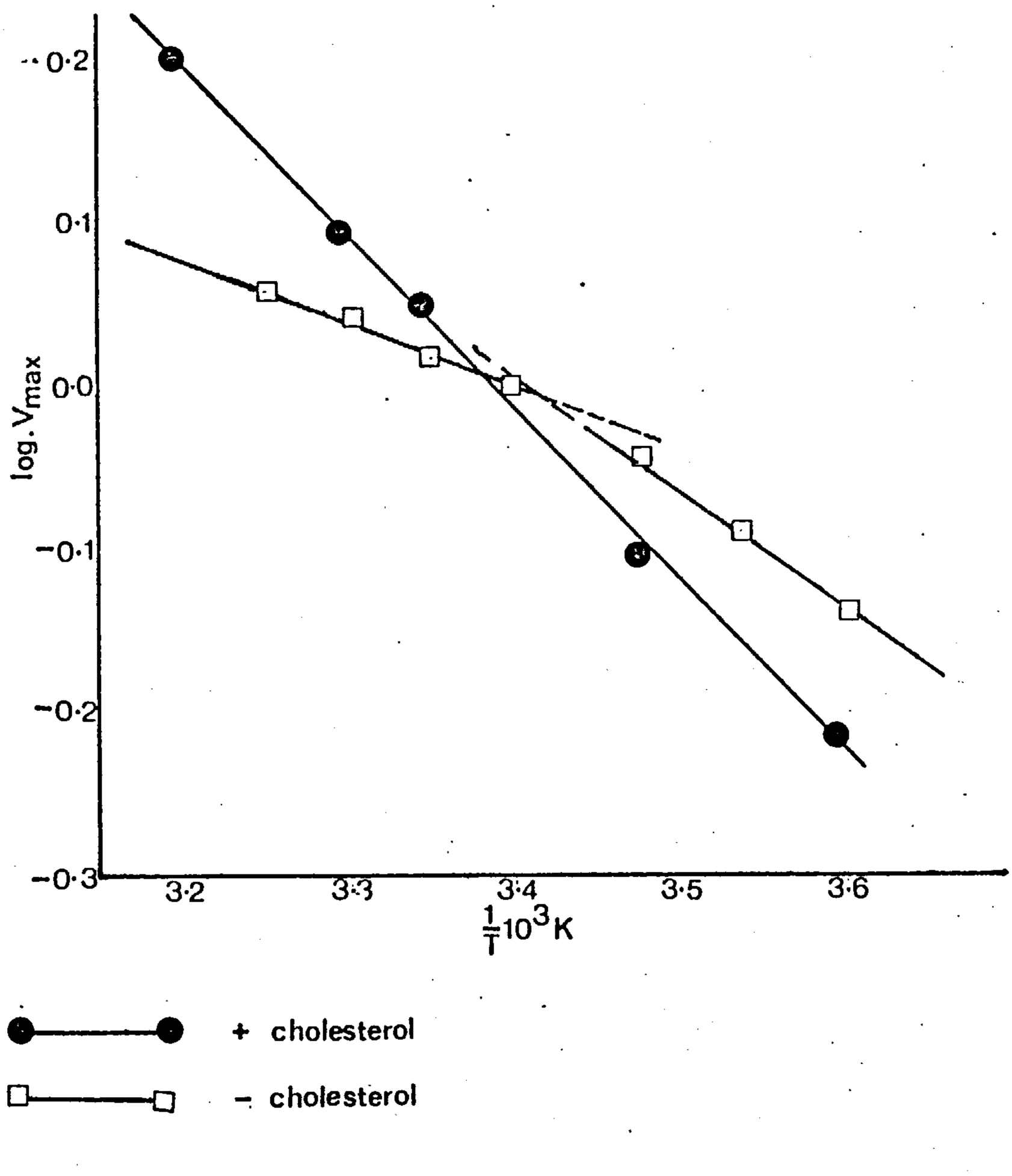
$$\Delta pKm(app) = 0{\cdot}43\ e\psi/kJ$$

(see introduction - Section 6.E)

it was possible to determine the membrane potential (ψ) at the liposome surface. This could then be compared with the "theoretical" value from a knowledge of the number of charged phospholipid molecules on the outer face of each liposome. (see also introduction - Section 6.E). Assuming that the area of each stearoylamine molecule was $1{\cdot}2nm^2$ (G.L.Gaines,1966),

Fig.III.23

Arrhenius Plots of Purified AChE Adsorped to Liposomes excluding or including Cholesterol



the theoretical value of ψ was calculated to be + 111 mV whereas the ψ derived from the experiments was found to be+ 9·41 mV. Myristic acid was also assumed to have an area per molecule of $\overset{0.6}{?}nm^2$. (Gaines, 1966) and the theoretical value of ψ was thus found to be -146 mV as compared to the experimentally determined ψ of -3·2 mV.

F. Effect of temperature on the binding of AChE to 50% dimyristyl PC/ 50% Myristic acid liposomes with or without cholesterol.

The effect of binding the AChE to dimyristyl PC/MA liposomes on either side of the transition temperature for dimyristyl PC was observed. At 25°C. the Kass was $9·5 \times 10^3$ mol^{-1} whereas at 4°C. this fell to $5·4 \times 10^3$ mol^{-1}. When cholesterol was incorporated in the liposome synthesis the Kass at 25°C. was 15xmol^{-1} falling to 3xmol^{-1} at 4°C. Table III.8. shows the AChE activity bound to the liposomes at 25°C. and 4°C. It is apparent from the table that lowering the temperature below the transition temperature or incorporating cholesterol in the phospholipid reduces the binding of AChE to the liposomes substantially below the level of binding to the liposomes without cholesterol at 25°C.

Table III.8

Effects of Lipid Composition and Temperature on the AChE Binding to Phospholipid Liposomes

Liposome Composition	Temperature $^{o}C.$	AChE Bound μ mol.min^{-1} mol.P lipid^{-1}
dimyristyl PC.50%/ myristic acid 50%	25	5.3×10^{-3}
dimyristyl PC.50%/ myristic acid 50%	4	2.9×10^{-3}
dimyristyl PC.49%/ myristic acid 49% cholesterol 1%	25	1.9×10^{-3}
dimyristyl PC.49% myristic acid 49% cholesterol 1%	4	2.0×10^{-3}

SECTION IV: DISCUSSION

DISCUSSION

The aims of the research presented in this thesis were to find a suitable method of bringing AChE into solution and then to purify the enzyme. Having obtained a very pure enzyme preparation, the next step was to examine enzymic forms in solution.

As a consequence of the mode of solubilization a further question was asked: What is the relationship of the AChE with the membrane ? Does the fact that the enzyme is bound to the outward facing aspect of the membrane mean that its relationship with the membrane is tenuous or is it bound fairly tightly ?

1. Solubilization

Although the efforts directed at bringing the enzyme into solution were really only an intermediate stage in the purification of AChE, it transpired that the varying successes of the different solubilizing agents had a bearing on the interaction of the enzyme with the membrane and served as an interesting rider to the actual membrane studies which are discussed later in this section.

When searching for a suitable technique for solubilizing AChE, the parameters borne in mind were particularly the yield of enzyme, the time spent in preparation and also the simplicity of the protocol. Yield of enzyme was considered to be the most important as many schemes for obtaining pure biological preparations are notorious for their ultimate low yield at the end of the process. It was therefore deemed necessary to make full use of the starting material. On these terms, the use of organic solvents, freeze-thawing, ultrasonication, low temperature lipid extraction and bacterial proteases have already been precluded by McIntosh (1973) on the basis of very low yields. He also rejects the use of lysolecithin as a solubilizing agent for routine use despite its high efficiency because of the high cost of this material. The following schemes were therefore tried bearing in mind those methods previously used for solubilizing AChE from different sources.

A. Dilute buffer or water

Incubation of pig brain homogenates in dilute buffer or water consistently gave yields of between 13% and 15% of the total activity (Table I.1) This figure agrees with the results of other workers who obtained very similar levels of AChE in this 'soluble' fraction. (Ho & Ellman, 1969; McIntosh, 1973; Devonshire, 1975). It was thought possible that this soluble AChE might in fact be in equilibrium with the enzyme which was present on the membrane and not a true soluble fraction. However, when the 100,000g pellet from this preparation was rehomogenised and incubated in buffer or water, no more AChE was brought into solution. This indicates that the AChE in the soluble fraction is a truly soluble pool of enzyme although its function is unknown. It is possible that this soluble enzyme originates from the membrane enzyme which is always being turned over but a satisfactory explanation has so far not been offered.

B. Chelating Agents

It is generally thought that EDTA facilitates the removal of proteins from the membrane by the disruption of divalent ion bridges which stabilize protein-lipid complexes. (Hollunger & Niklasson, 1973; Maddy & Dunn, 1973.) Several groups have therefore applied the use of EDTA to the removal of AChE from its membraneous matrix. (Chan, Shirachi & Trevor, 1970; Chan, Shirachi, Bhargava, Gardner & Trevor, 1972; Hollunger & Niklasson, 1973) and have solubilized between 50 and 70% of the enzyme from the particulate fraction. Using similar conditions to the above workers, it was found possible to bring 49% of the homogenate AChE activity into solution (Table III.2). The EDTA did not activate the enzyme in the homogenate. Although this was a substantial fraction of the total activity, it was not considered satisfactory enough to warrant using this method for preparing a soluble enzyme preparation for further use.

Chan et al (1970), who showed that 40% of the AChE could be solubilized from bovine brain caudate nucleus by EDTA, suggested from this observation that the brain enzyme might not be as firmly bound to the membrane as

some people had previously thought. Lerner et al (1973) extended this work by studying the effect of divalent cations on the efficiency of solubilizing AChE by EDTA and found that Ca^{2+} ions significantly prevented solubilization. This also agrees with the work of Silman & Dudai (1973) who showed that if Ca^{2+} was included in the medium when homogenizing electric organ from electric eel there was a large decrease in the AChE activity found in the 100,000g supernatant. Hollunger & Niklasson (1973) made it clear that this was not an ionic strength effect because equivalent concentrations of monovalent cations such as Na^{+} & K^{+} had substantially less influence on the solubilization of AChE. Hollunger & Niklasson (1973), by incorporating tetracaine into the EDTA extraction medium found that the AChE solubilization was improved and they explained this phenomenon by suggesting that the tetracaine displaced Ca^{2+} from its binding to phospholipids and/or proteins, and that this perturbation reduced the forces binding the enzyme to the membrane. A rise in pH has also been interpreted in this way. Hayden et al (1973) and Taylor et al (1973) explained the increased solubilization of AChE with increased pH as being due to a progressive reduction of the negatively charged sites on the carboxyl groups and phospholipid particles. At lower pH's these sites would form with divalent cations' co-ordinating covalent bonds with nucleic acids or electrostatic bonds with histones thus leading to higher molecular weight aggregates that sedimented with the membrane.

In the present investigation however, when tetracaine was incorporated in the EDTA incubation it was found that very little improvement of the total solubilization of AChE was achieved. (Table III.3). The only explanation that can be offered for this is that the above workers used bovine caudate nucleus as the enzyme source whereas in these studies the brain cortex from pigs was employed.

Varying the other conditions such as decreasing the ionic strength of the incubation media did not significantly increase the AChE recoverable in the soluble fractions. (Table III.3). EGTA gave a very disappointing

solubilization of the enzyme. No explanation can be offered for this phenomenon because this chelating agent has a higher affinity for Ca^{2+} ions than EDTA. It might conceivably be possible that the EDTA acts in a way other than removing the Ca^{2+} ions although this seems unlikely in the light of the results of Lerner et al (1972) and Hollunger & Niklasson (1973).

C. Autolysis

This method of solubilizing the enzyme was originally used by Rothenberg & Nachmansohn (1947) by storing parts of electric eel electroplax under toluene for several months. Dudai et al (1972) point out that with increasing time, the amount of 11S enzyme formed increases, possibly as a result of autolysis. Because the AChE is membrane bound it would be reasonable to hope that the proteolytic enzymes present in the brain homogenate would, to a certain extent, digest the membrane components thus releasing the AChE. However, it is a difficult process to control finely so that the proteases do not destroy the enzyme. Figs. III 4 & 5 show this was in fact the case in these studies. The autolytic process destroyed the greater part of the enzyme whether the brain cortex was homogenized before or after the autolysis and so this method was discarded as a way of obtaining a solubilized AChE preparation.

D. Tryptic digestion

Trypsin was tested as a solubilizing agent as other workers have used it with varying success to remove AChE from the membrane of electroplax from electric eel. As trypsin is a protease its solubilizing action is the same as described in the previous sub section for autolysis. Results however in these studies were very disappointing. When the trypsin treatment was allowed to proceed for more than 12 hours most of the AChE was invariably inactivated. If the conditions were readjusted so that the digestion was halted by soy-bean trypsin inhibitor after 4 hours of incubation, very little of the AChE was solubilized even though 95% of the total homogenate

enzyme was still active. Similar findings were also reported by Ord & Thompson (1951) who found that rat brain AChE was inactivated by Trypsin treatment.

E. Triton X-100

For proteins which are strongly associated with the lipid matrix of membranes the most useful method of extraction at present seems to be the use of detergents. (Helenius & Simons, 1975). The solubilization of AChE is no exception. Several groups of workers have successfully used detergents in the preparation of this enzyme. (Ho & Ellman, 1969; Wright & Plummer, 1972).

In these studies, the non-ionic detergent Triton X-100 was used following the method of Ho & Ellman (1969) with slight modifications. It can be seen from the results in Table III.6 that this procedure is very efficient in solubilizing the enzyme. A total of 56% of the homogenate activity was solubilized which represents over 65% of the enzyme present in membraneous structures. These results compare very favourably with those of McIntosh (1973) although Ho & Ellman themselves obtained yields of over 80%. The reason for this big difference is not certain. It is a well known fact that commercial preparations of detergents vary quite significantly with different amounts of additives and water being present. Also, Triton X-100 which is a polyoxyethylene p-t-octyl phenol has polydisperse polyoxyethylene head groups due to the statistical polymerization of ethylene oxide. Consequently the number of ethylene oxide groups per molecule stated by the manufacturer is only a mean value. (Helenius & Simons, 1975).

One disadvantage of using detergents in the solubilization of membranes is the difficulty with which they can be removed. Skangiel-Kramska & Niemierko, (1975) removed Triton X-100 from preparations of solubilized peripheral nerves by dialysis against deionized water. However, in work quoted by Wright (1971) an insignificant fraction of the Triton X-100 could be removed by this procedure. The reason for this is that this detergent has an extremely low critical micelle concentration (between 0·01%

and 0.02%) and consequently the molecule exists as micelles with molecular weight of about 90,000 daltons, (Kushner & Hubbard, 1954). In these studies therefore, a method formulated by Holloway (1973) was tested. This procedure relies on the specific adsorption of Triton X-100 to a neutral styrene-divinylbenzene polymer while the enzyme passes through unretarded. However, this method was found unsatisfactory for this brain preparation. Ott et al (1975) had similar difficulties in that they could not elute the erythrocyte enzyme from this resin in an active form. The method of Ott et al (1975) who used a column of hydroxylapatite to remove the detergent was found to be much more successful although a low amount of Triton X-100 was still required to be present to allow the enzyme to be eluted in sufficient yields and in an unaggregated form. If a completely detergent free elution protocol was followed, the enzyme remained bound to the column. Increasing the salt concentration in the elution medium had no effect on removing the enzyme until Triton X-100 was reintroduced into the column. When the detergent depleted enzyme prepared by Ott et al (1975) was passed through a column containing Sepharose 4B while excluding Triton X-100 from the elution media they found that more than 80% of the AChE remained bound to the column. They attributed this phenomenon however to the interaction of sugar residues in the enzyme molecule and carbohydrates in the Sepharose 4B gel.

Triton X-100 has been demonstrated to be a very useful agent for solubilizing membranes by several groups as this detergent does not seem to induce conformational changes in proteins leading to a loss of their characteristic properties. (Meunier et al, 1972 ; Rubin & Tzagoloff, 1973). Also, Triton X-100 appears to be very inefficient in disrupting the protein-protein interactions, (Helenius & Simons, 1975; see also later in this discussion) although cases of the quaternary structure of proteins being disrupted are known such as with haemocyanin (Helenius & Simons,1972) The fact that Triton X-100 is a non ionic detergent also means that it would be less likely to react with proteins by polar interactions such as might

happen with the anionic sodium deoxycholate. However, at the same time it has been apparently shown that Triton X-100 might be associated with hydrophobic areas on glycoproteins (Utermann & Simons, 1974). As it has been reported that AChE is a glycoprotein (Powell et al, 1973) and also has domains of a hydrophobic nature on the surface of the molecule (Steinberg et al., 1975) a reasonable prediction could be made that at least some detergent binds on to the enzyme. This factor would have to be considered when studying the properties of the detergent solubilized AChE such as an increase in sedimentation coefficient.

Having taken all these factors into account it appears that of all the solubilization procedures tested, extraction of the AChE from the membrane by Triton X-100 was the quickest and most efficient procedure of getting good yields of enzyme and seemed to be a suitable preparation for purification by affinity chromatography. It would have been preferable to have found a non detergent extraction procedure which was as efficient as that of Triton X-100 but this was not possible.

2. Purification

In order to study the properties of any biological molecule it is generally necessary to purify that molecule to a fairly high degree so as to exclude, as far as possible, any artefacts which could arise from the presence of impurities. In these studies this was particularly true for the investigation of the binding of AChE to lipid bilayers of the liposomes because phospholipids are very sensitive to small changes in environment.

In this department, pig brain AChE had previously been partially purified by a hydrophobic affinity column (McIntosh, 1973) which had been based on a procedure developed by Yon (1972). This column however had limited specificity for the enzyme due to the AChE being eluted with different combinations of salt and/or Triton X-100. In these present studies columns containing ligands specific for AChE were employed.

A. MAP-Agarose Column

This affinity column which was used originally by Goodkin & Howard (1974) to purify the AChE of rat brain synaptosomal plasma membranes was adapted for use on the pig brain cortex enzyme. The attraction of this method was that Triton X-100 had been used to solubilize the rat brain AChE and so a procedure was available which involved the presence of detergent in the elution media.

The preliminary purification of the Triton extract from brain (Fig.III.1) demonstrated that both AChE and other proteins were retarded on the MAP-Sepharose column when applied in the presence of Triton X-100. The fact that the detergent was present indicated that it was unlikely that the enzyme was being retarded by hydrophobic interactions on the apolar side chain.

When a gradient of sodium chloride was applied, two small peaks of protein and enzyme activity were removed from the column at approximately 100 mmol/l and 350 mmol/l NaCl. It is possible that this was due to the Sepharose matrix acting as an ion exchange column. This has been suggested previously by O'Carra, (1974). In later experiments when Triton X-100 was excluded from the elution media however, no AChE was eluted which suggested that any non-specific enzyme binding was due to hydrophobic and electrostatic interactions acting in concert with the column. This phenomenon was also noted by McIntosh (1973) who showed that both detergent and salt were required to release AChE from a hydrophobic affinity column.

The specific AChE inhibitor, edrophonium chloride removed 31% of the total enzyme from the column when it was applied after the salt gradient and the overall purification obtained was 5 fold. This did not compare very favourably with the results of Goodkin & Howard (1974) who purified the rat brain enzyme by 100 to 150 fold. The reason for this difference in these purifications was not discovered. It might have been that there were definite differences in the properties of the rat brain and pig brain cholinesterases. However, this degree of purification was not considered

to be efficient enough to allow further detailed study of the enzyme.

When the MAP-Agarose was subjected to a final wash with 1 mol/l NaCl, between 30% and 40% of the total AChE was eluted. As this fraction was not removed by the edrophonium chloride, it seemed that it had been binding to the column non-specifically. It seemed likely that this interaction with the column was by electrostatic interaction because when the 0-400mmol/l salt gradient was excluded the corresponding peaks of AChE activity appeared in the 1 mol/l NaCl peak. Also the omission of this salt gradient had very little effect on the purification of the enzyme in the edrophonium chloride peak (Table III.7). It is always very likely that when an affinity column is constructed in several steps such as with the MAP-Sepharose unreacted free groups are left which do not couple with the ligand. In this case it is possible that there were free carboxyl groups which had not reacted with the N-methyl-3-aminopyridinium iodide (Fig.II.3) This would have explained why the affinity resin might have had ion exchange properties.

B. ε-Aminocaproyl-PTA Agarose column

As the MAP-agarose column was very disappointing in its purification of AChE, the phenyltrimethylammonium ligand was selected as it has been proved to be a good competitive inhibitor of AChE especially when bound to an ε-aminocaproyl side-arm. Dudai et al (1972) have shown this moiety to have a Ki of 6μmol/l for the enzyme.

The initial experiments with pig brain AChE on this column were very disappointing (Fig.III.3) Only 0.4% of the total AChE applied came off in the edrophonium chloride wash with no significant purification after dialysis. The 1mol/l NaCl peak removed 22% of the enzyme but here again there was no purification because contaminating protein which had bound non-specifically to the column was removed in this wash. By increasing the edrophonium chloride concentration it was thought that the enzyme might have been eluted but even a concentration 50 mmol/l failed to achieve this. It was thought possible from this observation that the AChE might have remained on the column by binding on to the quaternary nitrogen atoms

by way of one of its peripheral anionic sites as well as by the active centre anionic site. Thus the edrophonium chloride which has only one positively charged nitrogen atom would have had no effect in dislodging the enzyme. For this reason, another competitive inhibitor - decamethonium bromide - was used in an attempt to elute the AChE. This molecule was chosen because it has two positive centres separated by a decamethylene bridge which is just the correct dimensions to span the distance between the active site anionic site and peripheral anionic site. Also several other groups seem to have preferred to use this inhibitor in the affinity chromatography of AChE (Dudai et al., 1972). Fig.III.4. shows the elution profile of AChE eluted from the ϵ-aminocaproyl-PTA column with decamethonium. The decamethonium peak after dialysis gave a purification of 16 fold with a recovery of 35%. This was a definite improvement on the MAP-agarose column and also the ϵ-aminocaproyl-PTA column using edrophonium but it still did not approach that of 700 fold by Chan et al.,(1972)[a].

An attempt which was made to obtain a detergent free AChE preparation by eluting with decamethonium in the absence of Triton X-100 failed because the enzyme was quantitatively adsorped to the Sepharose matrix in a similar manner to the MAP column. This was probably due to hydrophobic interactions of the enzyme with the apolar part of the ligand separating the quaternary nitrogen group from the agarose backbone. Even increasing the salt concentration or the eluting inhibitor concentration failed to release the enzyme. However, it was found possible to obtain a comparably pure preparation of the AChE if Triton X-100 was included to a concentration of 0.1% w/v and eluted with the decamethonium bromide.

Although the enzyme was not purified sufficiently to warrant using this column as a standard procedure there was no doubt that the ϵ-aminocaproyl--PTA agarose were very efficient in retarding the enzyme. This was shown by the fact that large volumes of the enzyme could be applied without being eluted by the buffer. The largest volume of enzyme used was 300ml. which contained approximately 150 units of AChE activity, and even with this load

very little enzyme came off the column although contaminating protein was washed off. The only thing limiting the volume of AChE applied was getting enough starting material. It would have been interesting to have discovered the saturating concentration of AChE on the column. Also it was advisable not to use too high volumes of the crude AChE preparation because this would have increased the time of elution thus increasing the risk of proteolytic digestion of the enzyme.

It was not possible to increase the flow rate of the enzyme through the column for the following reason. Rosenberry & Bernhard, (1971); Rosenberry et al, (1972) have measured the competitive inhibition dissociation constants (Ki) of free ϵ-aminocaproyl-PTA and also of the same ligand bound to Sepharose. They found no significant difference in the two values. However, they did find a significant difference in the time it took to attain steady-state inhibition. The rate for the unbound ligand was almost instantaneous whereas for the Sepharose bound inhibitor, the steady-state situation was only reached after approximately 30 min. They attributed this to a low bimolecular collision frequency between enzyme and Sepharose 4B beads leading to enzyme immobilization. Thus if the AChE were to be run through the column fairly fast it would have been probable that some enzyme would have been eluted together with the contaminating protein without being retarded.

C. MAC-Agarose column

The MAC-Agarose column which was originally developed to purify the aggregating species of AChE from electric eel proved to be the most successful column tested for the purification of the pig brain enzyme. As with the ϵ-aminocaproyl-PTA Sepharose, up to 250 ml. of the crude AChE preparation could be run through the column without the enzyme passing through unretarded indicating that the enzyme was binding to at least one site on the affinity column.

The enzyme eluted from the MAC-agarose with decamethonium was found to be 901 fold purer after dialysis than the applied enzyme, with a 44% yield

and activity of 148 μmol.min,$^{-1}$ mg^{-1}. This compared very favourably with the results of Dudai et al.(1972) who eluted 50% of the electric eel AChE from the same column but in the presence of high ionic strength NaCl (1 mol/l). When the brain preparation was rechromatographed on the column, the specific activity was at least double although the protein content could not be determined accurately due to its low level.

When preliminary experiments were tried where high ionic strength NaCl (1 mol/l) was included in the buffer, as done by Dudai et al.(1972), only very small amounts of the enzyme were retarded whereas the above group had great success in binding the AChE to the column under high ionic strength conditions. It was possible that the difference in results was due to the enzyme originating from separate species and also that the eel enzyme was detergent free whereas the brain enzyme was in the presence of Triton X-100. However, the purification scheme used in this work was very successful if the enzyme was eluted with decamethonium bromide at low ionic strength.

The fact that the high ionic strength elution removed more AChE from the column after the decamethonium wash (Fig.III.5) indicated that there was some AChE that had bound to the column non biospecifically probably by electrostatic interactions. These interactions might possibly have been due to the hydrophobic macroreticular matrix of Sepharose. Also when the Triton X-100 was completely excluded from the elutions the enzyme was quantitatively adsorped to the Sepharose matrix as with the other two affinity systems. It was probable that the detergent depleted enzyme in this case bound hydrophobically to the apolar side chain separating the quaternary nitrogen on the MAC from the Sepharose backbone. If only 0·1% w/v Triton X-100 was reintroduced into the elution procedure with decamethonium bromide the enzyme was removed and this was indeed the method used for preparing a low detergent sample of the AChE for further use.

The presence of hydrophobic and electrostatic interactions in chromatographic procedures can present even greater difficulties in hydrophobic interaction chromatography. In this technique, the purification of the

molecule in question depends solely on the adjustment of elution medium so that the hydrophobic and electrostatic forces are reduced to a level to allow the removal of that specific molecule. (For a review see Morris, 1976).

3. Multiple Molecular Forms

Maddy & Dunn (1973) have stressed the warning that as any protein which is extracted from its native membrane undergoes a change in the environment of that protein, all extraction methods must be suspected to at least some extent of producing aggregates which do not exist in the native membrane. Therefore, any solubilized fraction might conceivably be an aggregate of biologically dissimilar molecules of which only some might be responsible for the biological activity under study. It was therefore of great importance to bear these facts in mind in this work when drawing conclusions about the molecular weight of the various multiple molecular forms of AChE from pig brain.

In order to rule out the possibility of these anomalies it was advisable to use several different extraction procedures and several more techniques for the characterisation and the separation of the molecular forms of the enzyme. Thus, if molecular species which were prepared in different ways and resolved by different means appeared to be very similar, it would have been reasonable to assume that the different molecular forms were not artefacts of experimental procedure.

The three techniques of molecular separation used in this work - polyacrylamide gel electrophoresis, sucrose density gradient centrifugation and starch block electrophoresis - have all been used widely in biological investigation and have shown to give reproducible results.

A. Sucrose Density Gradient Centrifugation

This technique was at first used with the intention of separating the molecular forms of AChE in sufficient quantities for further investigation. However, the difficulty arose that when large amounts of protein were

applied to the sucrose gradient and centrifuged the linear relationship between the migration of the molecule down the tube and the sedimentation constant of that molecule was lost. This effect was noted by Steensgaard, Møller & Funding (1975) who showed that migrating zone mass centres were dislocated, zone shapes were changed and even individual protein zones split into two zones when gradients were overloaded. However, when the amount of protein applied to the gradient was less than 50mg. per gradient, the linear relationship was retained. (Fig.III.6). This result agrees with those of Martin & Ames (1961) and also complies with the Steensgaard et al. (1975) provisions about overloading. It was therefore concluded that this method would be suitable for use in the analysis of the multiple forms of AChE.

The fresh preparations of Triton solubilized AChE which had not been purified whatsoever when run on sucrose gradients (Fig.III.7) always showed one molecular species with a sedimentation constant of 11-12S. This value approximates to a molecular weight of 240,000 which shows a close correlation to estimates reached in other laboratories. (Leuzinger et al., 1968, Hollunger & Niklasson, 1973). The same result was obtained when 1 mol/l NaCl was incorporated except that the peak of enzyme activity was sharpened up considerably (Fig.III.8). When the five day old enzyme was centrifuged on gradients, no peak of activity could be resolved. Instead there seemed to be a smudge of activity throughout the gradient. It was possible that the enzyme had aggregated to different multiples of the 240,000 species, and what should have been resolved into individual peaks was spread throughout the gradient. This phenomenon of aggregation has been reported previously for brain acetylcholinesterase from calf (Hollunger & Niklasson, 1973, Viana et al., 1974). The latter authors indeed showed gradual aggregation of the lower molecular weight species through the intermediate molecular weights up to the large aggregates. Hollunger & Niklasson (1973) have suggested that the aggregation might have been brought about by the enzyme being transformed into an aggregating form by proteolytic

enzyme action or non enzymic oxidation of SH-groups. They discount this however on the grounds that they could partially disaggregate the enzyme by treatment with DEAE-Sephadex. But, in this work it was found that treatment of the aged AChE preparation with DEAE-Sephadex caused no change in the state of aggregation. The same authors also offer the alternative possibility that the AChE released in the presence of DEAE-Sephadex did not aggregate because some factor such as a phospholipid (Grafius, Bond & Millar, 1971) or a small protein (Kremzner & Fei, 1971) might have been adsorped to the ion exchanger. However, as far as work in this laboratory is concerned with pig brain this is unlikely because as well as the failure of DEAE-Sephadex to stop the AChE aggregating, the affinity chromatography purified enzyme showed exactly the same phenomena on the density gradient as the crude preparation. If an aggregating molecule had been present with the enzyme molecule, the process of purification on the affinity column would have presumably removed it thus preventing the characteristic aggregation.

The 'soluble' preparation of AChE also showed a major molecular form of 11-12S and also a minor species of 15S & 19S which corresponded to molecular weights of 360,000 and 525,000 respectively, (Fig.III.9). The same pattern was also true of the EDTA solubilized enzyme (Fig.III.10). Both of these preparations showed the same tendency to aggregate although the degree of aggregation was very variable from one preparation to another. The reason for this was unknown.

As the process of aggregation by the various molecular forms had been shown to occur in other laboratories, it was interesting to note that when the 11S peaks from the above mentioned preparations were recentrifuged on gradients, sedimentation characteristics remained unchanged whereas when the 15S or 19S peaks were re-run there was found to be a range of species from 10S to 20S. In fact it seems that there was a general process of aggregation and disaggregation. These findings appear to agree quite closely with those of Rieger et al., (1972), Dudai et al., (1973) who

showed that the 14S & 18S species of electric organ AChE from electric fish did not aggregate at low ionic strength. These two molecular species might be compared with the 15S & 19S molecules found in the solubilized pig brain cortex enzyme. One major difference however was that although their 11S AChE did not aggregate, in this work the 11S Triton solubilized enzyme did show this process. This may have something to do with the fact that whereas the electric organ AChE seems to be much more tenuously associated with the membrane as demonstrated by the degree of ease with which it may be released, the brain enzyme is much more difficult to remove as can be seen from the varying success of solubilizing agents tested in this work. The solubilizing of the enzyme might have caused in the enzyme a tendency to aggregate, (Maddy & Dunn, 1973).

It would have been interesting to have determined the effect of trypsin on the gradient profiles and the aggregating properties of the enzyme. The drawback with this is that the AChE from brain is rapidly denatured by this treatment (see Ord & Thomson, 1951, and this thesis). Dudai's group have shown that proteolysis with this enzyme quantitatively transforms all the molecular forms of electric eel AChE into the non-aggregating 11S form. (Dudai et al, 1972). Autolysis has also been shown by the same author to convert the eel AChE to the 11S form but the brain enzyme was destroyed by this procedure.

The results showing the sedimentation properties of the starch block electrophoresis AChE gave very consistent findings. The fact that the one peak from the Triton-solubilized AChE present on the starch block containing no detergent migrated to the bottom of the tube indicated a very highly aggregated state of the enzyme. The AChE corresponding to Peak I on the starch block containing detergent must also have been highly aggregated as this showed the same phenomenon. This process of aggregation of the Triton depleted enzyme on density gradients has also been reported for human erythrocyte acetylcholinesterase by Ott et al., (1975). Peak II of the AChE on the starch block containing detergent however,

showed exactly the same sedimentation properties as the non-electrophoresed enzyme. The pattern of an aggregating and a non-aggregating species of brain AChE was also shown by the soluble AChE from the starch block. Therefore it seemed that the molecular dimensions of the enzyme were very similar whether naturally soluble or solubilized by Triton, provided detergent was present in the Triton preparation. This conclusion was also arrived at by Ott et al., (1975) for the human erythrocyte AChE. Tanford, Nozaki, Reynolds & Makino (1974) have stated that membrane proteins probably retain their in vivo conformation if solubilized by mild detergents such as Triton X-100.

An apparently anomalous result met by Ott et al., (1975) where the Triton solubilized enzyme 'floated' in the sucrose gradient giving an apparently low sedimentation constant, was not found in this work. They explained their results by saying that the protein molecule probably combined with detergent molecules which floated the enzyme in the sucrose. Tanford et al., (1974) have said that Triton X-100 most probably binds to lipophilic zones on the enzyme thus increasing the partial specific volume. Possibly the floating phenomenon was not met with the brain enzyme because not so many detergent molecules bound to the protein.

B. Polyacrylamide Gel Electrophoresis

i. Polyacrylamide Rods As a technique taken by itself Davis' (1964) disc electrophoresis method tells us little about the molecular weight of the different molecular species of proteins. However, it does offer a method of separating high molecular weight substances such as proteins, and in this work, of showing up differences in the molecular species prepared by different procedures.

The electrophoretic patterns of the AChE in this work is presented in Fig.III.11. It can be concluded from the electropherograms that there were very many similarities between the AChE solubilized by the several different methods. The 'soluble', 'Triton solubilized' and 'EDTA' solubilized AChE had molecular forms which migrated to corresponding distances

down the gels, although the Triton solubilized AChE had slightly greater activity at the cathode than the other preparations.

There was always found to be some surface staining on the gels even when the polyacrylamide contained 1% w/v Triton X-100. This observation conflicts with that of McIntosh (1973) who found Triton to facilitate entry. He explained his results by saying that protein aggregation could account for the enzyme not entering the gels. From this point of view this was similar to the present findings as the slow moving peak (I) from the starch block electrophoresis (Triton included) of detergent solubilized enzyme only just entered the gels even though the slow moving peak showed substantially the same pattern as the crude Triton solubilized enzyme. The same findings were also true of the 'soluble' preparation of AChE. It was interesting to note that the aggregated AChE would not enter the polyacrylamide even when Triton X-100 was included in the gel. Thus it appeared that the aggregation was unable to be reversed by the presence of Triton X-100. Dudai et al, (1973) could disaggregate the 18S & 14S molecular forms of eel AChE by converting them to the 11S forms by maleylation but they don't mention whether the aggregated 18S and 14S forms could be disaggregated without being transformed to the 11S molecular form.

McIntosh (1973) showed that in this electrophoresis set-up, charge differences in the molecular forms were not the only factors for separating the isoenzymes because when the pore size of the polyacrylamide was increased and the time of electrophoresis kept constant, protein migration increased but two of the bands merged into one. He also noted that when the run was extended to 3h., two bands again appeared instead of one. The two most likely reasons he cites for this are: an aggregation-disaggregation phenomenon or alternatively, after the 1½h. run in an increased pore size gel, the differences in molecular weight are counterbalanced by differences in charge giving an apparent single molecular form. The second reason is more likely because aggregation as stated above appeared in this enzyme preparation to be an irreversible process and when McIntosh extended the

electrophoresis run, the two bands reappeared.

The fact that the different preparations of enzyme including the affinity purified AChE showed very similar band patterns and also resembled those obtained by McIntosh (1973) went a long way to concluding that the extraction procedures did not cause artefacts. Also, the band patterns shown were indeed acetylcholinesterase as the inhibitor BW284C51 abolished the specific staining.

ii. Polyacrylamide Slabs Electrophoresis on gel gradients when run to equilibrium has the advantage over the previous method of separating molecules almost exclusively by molecular size and allows us to estimate the molecular weights of the multiple molecular forms. In this case it also means that the molecular weight value obtained can be compared with those attained by sucrose density gradient centrifugation.

The principle involved with this technique is that the proteins pass through a gradient of progressively smaller pores in the polyacrylamide until they reach the point where the size of pore restricts their passage. Here the proteins concentrate and can be detected by staining. A great advantage of the technique is that several proteins can be run side by side on the same gel, thus allowing the direct comparison of the proteins. This also removes any inaccuracies caused by differences between individual gels.

The band patterns obtained for the standard proteins showed a straight line relationship when migration distances were plotted against the $\log_{10}$ M.W. (McIntosh, 1973). Fig.III.12. shows the patterns of molecular forms obtained by the electrophoretic separation of AChE by this technique. The one common feature which appeared between all the solubilized preparations was a molecular form with a MW of approximately 250,000. This agreed very closely with the approximate M.W. (240,000) found by sucrose gradient centrifugation and also by McIntosh & Plummer (1973) who found molecular weights of between 245,000 and 288,000 for seven different preparations of the enzyme. Hollunger & Niklasson (1973) also found one of the molecular

forms of AChE from calf brain caudate nucleus to have a M.W. of 250,000 but Viana et al (1974) arrived at a M.W. of 219,000 using a very similar extraction procedure and exactly the same source.

The lowest M.W. obtained here was 68,000 daltons which although higher than that obtained by McIntosh & Plummer (1973), was of the same order. It is tempting to extrapolate this to being the monomer of the enzyme and the 250,000 molecular weight species was a tetramer composed of four of these subunits. Leuzinger et al, (1968) also drew the conclusion that the tetramer had a M.W. of 240,000 composed of subunits of 64,000. Dudai & Silman (1972), however found two types of subunit of M.W. 59,000 and approximately 85,000 and they said that the smaller unit was an autolysis product from the larger one which in turn was associated into a tetramer of M.W. 320,000-350,000. It was possible that the brain enzyme could exist as a tetramer of the autolysed subunit. This was in fact how Dudai & Silman (1973) explained the results of Leuzinger et al. (1968) and this is possibly what happened with the brain AChE. The gradient electrophoresis of the Triton solubilized AChE did in fact usually show a very faint band of activity corresponding to a M.W. of 83,000 as well as the stronger staining 68,000 band. In addition this same preparation showed staining at a point corresponding to a M.W. of 365,000. Thus it is likely that the Triton solubilized AChE exists as a tetramer or monomer with or without smaller polypeptides being autolysed off the molecule. If the autolysed and unautolysed subunits existed side by side, the other band in the Triton solubilized preparation corresponding to a M.W. of 181,000 could have been a trimeric assembly of the 68,000 dalton subunits or a dimeric assembly of the 83,000 dalton subunit.

The 'soluble' extract of AChE never showed an 83,000 molecular form or a 181,000 form, but did show a band at M.W. 135,000. It is not known what the significance of the soluble AChE is physiologically. If it was a product of the natural turnover of the membrane bound AChE this might tie in with the fact that all the monomeric AChE in the soluble fraction was

of a M.W. of 68,000 having been autolysed. This, however, is only speculation.

EDTA extraction showed four bands of activity on gradient gels including the characteristic one showing a molecular weight of 240,000. Surprisingly however, the lowest M.W. form was 84,000 and there was no trace of the 68,000 subunit. It appears from these findings that EDTA inhibited the autolysis, but on this basis it is difficult to explain why a band of activity appeared with a M.W. of 240,000.

Gradient electrophoresis of the peaks of activity eluted from the starch block electrophoresed AChE showed similar results as the polyacrylamide rod electrophoresis of the said peaks. Thus the slow moving peak from the starch block only just entered the polyacrylamide and the fast moving peak showed similar staining as the crude extracted enzyme. This backs up the idea of the presence of both an aggregating and non-aggregating population of multiple molecular forms.

The fact that the non-aggregating molecular forms of Peak II showed similar electrophoretic patterns as before starch block electrophoresis indicated that the differences in multiple molecular forms were mainly due to differences in molecular weight rather than charge.

The most surprising results with the gradient electrophoresis were with the affinity chromatography purified enzyme. Here there were five bands of activity of M.W's between 68,000 and 250,000. It is possible that the process of affinity chromatography was just long enough to allow selective autolysis of the molecular species. Also, the actual bands of staining were very much sharper for the purified than the crude 'Triton solubilized' AChE. This was to be expected as there would be less likelihood of contaminating proteins binding to the AChE molecular forms causing an increase in range of molecular weight.

The lowest molecular weight in this work therefore was taken to be 68,000. This correlates well with the results of Gentinetta & Brodbeck (1972) who also found a M.W. of 68,000 for AChE. Lower M.W's have been found by other

researchers but they have had no enzyme activity. (Hillar & Grafius,1970).

C. Starch Block Electrophoresis

This technique, although rarely used nowadays, was employed for these studies so that any molecular forms which might have differed in charge could have been separated. Also, because it is a semi-preparative procedure, any separated enzyme can be further analysed.

Fig.III.13. shows the profile of the Triton solubilized AChE when the detergent was excluded from the block. Only one major peak was resolved whereas if Triton was present in the block, two peaks were resolved (Fig.III.14). As the removal of Triton was shown to cause aggregation of Triton solubilized AChE it seems that the non-resolution of the enzyme on Fig.III.13. was due to this aggregation phenomenon especially as the presence of Triton caused splitting of the peak. The slow moving peak (Fig.III.14) was also shown to be in an aggregated state as already shown by polyacrylamide gel electrophoresis.

These same phenomena were shown by the affinity chromatography purified enzyme.

'Soluble AChE' always showed two peaks of activity even in the absence of Triton X-100, and as already exemplified by polyacrylamide gel electrophoresis, the slow moving peak was an aggregated form and the fast moving peak an unaggregated form.

One anomalous result was the occasional appearance with the crude Triton-solubilized AChE of a small peak of AChE activity at the anode. The presence of this apparently very highly charged species of enzyme molecule cannot be explained. When the electrophoresis time was reduced the peak disappeared. It could only be dismissed as an artefact.

The general findings of this separation technique were that the differences in the molecular forms were not due to differences in their charges. This indicates that the separation of AChE forms on polyacrylamide gel rod electrophoresis was due to differences in molecular weight exclusively.

4. Studies on Membrane Associated AChE

A. Arrhenius Plots of AChE preparations

The Arrhenius plots of the membrane AChE clearly showed a break at 27°C. The Activation energies on either side of this temperature were very different being quite low at 8·3 kJ/mol. above this temperature and much higher at 39 kJ/mol. below this temperature. There are many reasons for the existence of such phase changes at specific temperatures with enzymes and Dixon & Webb (1964) have cited several explanations for such discontinuities. For example, phase changes in the solvent have been seen with lipase; sucrase exists in two forms of differing activities and the enzyme changes from one form to another at 22°C. In these studies with AChE however, the biphasic straight line became monophasic on the addition of Triton X-100 and the single activation energy for the enzyme was found to be 20 kJ/mol. Similarly, the 'soluble' preparation of the enzyme showed no phase change and had a similar activation energy of 19 kJ/mol.

Thus, it is very possible that the phase change which was only shown with the membrane bound enzyme reflected its status as a membrane bound protein. This conclusion however is certainly not unequivocal. The break in the Arrhenius plot could have been attributed to a local structural change rather than a true phase change in the integral membrane. (see questions and answers in Plummer et al, 1975)

Ciliv & Özand (1972) made studies on the Arrhenius plot of erythrocyte AChE and found a phase change at 32°C. with a soluble preparation. They mention the possibility that the break was due to a change of the aggregation state of the enzyme or by a reversible change of enzyme protein between two states indicating that one of the states was more active at temperatures above 32°C. This phase change was certainly not detected however for the soluble preparations of brain AChE. (Figs.III.17 & III.18). It seems that this difference in results might have been due to a difference in the source of enzyme.

Because the results of this work indicated that a phase change in the actual membrane may have been responsible for causing the two distinct activation energies in the membrane bound enzyme, it was thought that if the solubilized enzyme was bound to an artifical membrane and still showed a phase change characteristic of the constituent phospholipids, it would provide further evidence of the association of AChE with the membrane <u>in vivo</u> (see Section IV.3.B)

B. <u>Effect of Cholesterol on the Arrhenius plots of Phospholipid Membrane bound Acetylcholinesterase</u>

When lipids are heated they do not pass from a crystalline form to liquid in a direct manner and thus to vapour, instead they exist in an intermediate state commonly referred to as mesomorphic, liquid crystalline or anisotropic liquids. The temperature at which the lipid passes from crystal to liquid crystalline form is the transition temperature and is characteristic for each lipid (Hauser, 1975). The transition temperature is a function of hydrocarbon chain length, the presence of cis or trans double bonds and also the nature of the head group. At crystalline temperatures the hydrocarbon chains appear to be frozen in a planar conformation but as the temperature rises there is a concommitant increase in the oscillations and rotations about the hydrocarbon C-C bonds. Above the transition temperature the methylene groups of the hydrocarbon sidechains undergo rapid oscillatory, rotational and translational motions. (Hauser, 1975). The 'melted' chains partially fold back on one another reducing the end to end distance thus leading to an increased cross-sectional area and a 'thinned' bilayer.

The effect of incorporating cholesterol into the bilayer is to severely reduce the motional freedom of the methylene groups of the hydrocarbon chains, (Chapman & Penkett, 1966) There is also a concommitant increase in the bilayer thickness due to the closer packing causing the chain axes to become perpendicular to the plane of the bilayer. Thus the condensing effect of cholesterol tends to favour the crystal phase of the lipid bilayer.

The phospholipid dimyristyl phosphatidylcholine has been reported to undergo a phase transition at about 24°C. (Redwood & Patel, 1974). The transition temperature found here for AChE bound to this phospholipid bilayer was found to be in the same range at approximately 21°C. Also when cholesterol was incorporated in a 1% mol/mol ratio the break was abolished. These findings could be interpreted in the light of the above discussion; the cholesterol condensing the phospholipid bilayer so as to keep it in its crystal form even above the transition temperature characteristic of dimyristyl phosphatidylcholine. Thus the break in the Arrhenius plot of AChE was probably due to a true phase change in the integral membrane and not a local structural change.

C. Effect of Liposomes on Km of purified AChE

The effects caused by charged liposomes on the Km of AChE can be explained by interpreting the pH changes at a surface in relation to the bulk phase (Wooster, 1975), and unequal distribution of substrate between the enzyme-polyelectrolyte conjugate and outer solution (Katchalski, Silman & Goldman, (1971).

Acetylcholinesterase contains in its active site an anionic or negative centre (Wilson & Bergmann, 1950a). This area on the molecule is responsible for orientating the ester bond in the acetylcholine over the esteratic site by binding the quaternary nitrogen of the choline on to the anionic site. On the basis of charge of substrate and its effect on enzyme binding, the mode of interaction between acetylcholine and AChE appears to be ionic (Hestrin, 1949; Wilson & Bergmann, 1950b). These workers found decreased binding of positively charged substrates to the enzyme as the pH was raised. Similarly, the lowered affinity of substrate for a negative surface would therefore result in an increased Km of the enzyme for acetylcholine. With parallel reasoning, a decreased pH at a surface would favour the positive charge on the acetylcholine and result in a decreased Km for the substrate with the enzyme.

In this case, the negatively charged liposomes composed of 30% myristic acid and 70% phosphatidylcholine and the positively charged liposomes composed of 15% stearoylamine and 85% phosphatidylcholine, were used for the charged surfaces. In the case of the negatively charged lipid bilayers, the pH would have been lower than the bulk solution as hydrogen ions would have been attracted to the surface. Conversely, the opposite was true where the pH at the surface of the positively charged liposome would be higher than the bulk solution.

When the actual results were analysed it could be seen that the positively charged liposomes did indeed substantially raise the Km of acetylcholine for AChE to 150 μM as compared to 44 μM in free solution. On the other hand, when the enzyme was bound to the negatively charged liposomes, the Km was 29 μM. In both of these cases the addition of Triton X-100 abolished an increase or decrease of Km thus underlining the effect of the polyelectrolyte nature of the liposomes on AChE hydrolysis.

The formation of a pH gradient across the diffusion layer is very pronounced with AChE due to its very high turnover number, calculated to be $7{\cdot}4 \times 10^5$ min.$^{-1}$ per active site by Wilson & Harrison (1961), and in the case of membrane fragments obtained from homogenised electric organ tissue, the local pH in the vicinity of the membrane bound enzyme was as much as 2·5 pH units lower than in the bulk solution. (Silman & Karlin, 1967).

D. Effect of Temperature on Adsorption of AChE to Positively and Negatively charged Liposomes

Results in section IV.3.C. showed from the Scatchard plots the Kass from which could be obtained the apparent free energy change $\Delta G'$ for the interaction. In the case of both the positively charged and negatively charged liposomes there was very little change in the association when the temperature was lowered from 25°C. to 4°C. The apparent enthalphy change calculated from the Van't Hoff equation was therefore small at -8kJ/mol for the positively charged liposomes and -5 kJ/mol for the negatively charged liposomes.

However, the corresponding entropy calculated from the Gibbs-Helmholtz equation was large:

$$\Delta G = \Delta H - T\Delta S$$

The entropy change ΔS for the positive liposomes was + 70 kJ mol^{-1} K^{-1} and the ΔS for the negative liposomes+69 kJ.mol^{-1} K^{-1}. Redwood and Patel interpreted the large entropy change as providing the driving force for the formation of a liposome-ATPase complex and said this was indicative of hydrophobic interactions between the lipid and protein molecules (see also Kauzman, 1959). The presence of hydrophi~~e~~obic interaction would indeed lend support to the idea that AChE was an integral protein interacting with the apolar interior of membranes in vivo.

The results in Table III.8. showed that two factors affected the binding of AChE to dimyristyl phosphatidylcholine liposomes. These factors were the lowering of the temperature of binding below the transition temperature 24°C. or incorporation of cholesterol into the phospholipid. This was probably due to the same reasons as mentioned in Section IV.3.B.: that there was a reduced fluidity of the hydrocarbon chains upon the addition of cholesterol or a reduction in temperature. Papahadjopoulos, Cowden & Kimelberg (1973) have also reported a decrease in protein binding to phospholipid liposomes after the incorporation of cholesterol.

E. Membrane Potential and AChE adsorption

The difference between the 'calculated' and the 'theoretical' values of the membrane potential arise from the fact that the theoretical ψ does not allow for the effects of protein adsorption. From the value of Km obtained for AChE bound to positive liposomes, the surface potential was calculated to be +9.41mV. which was much lower than the theoretical value of +111mV. Similarly, with the negative liposomes the surface potential was found to be -3·2mV which again was lower than the theoretical value of -146mV. Thus it seems that the binding of the charged lipids by the

protein AChE lowered the surface potential.

Is then AChE an integral protein ? Singer (1974) has laid down several criteria for distinguishing integral proteins from peripheral proteins. These include the requirement of detergents to dissociate them from the membrane and also the usually aggregated or insoluble state of the protein when in neutral aqueous buffer. Results in this thesis have shown that Triton X-100 was the best solubilizing agent for AChE and when the detergent was removed leaving the enzyme in aqueous buffer, the AChE aggregated. Also, the fact that Triton X-100 was required in the elution media of the affinity chromatography protocols and that there was entropic contribution to the Gibbs free energy, pointed to AChE being an integral protein. Having stated his criteria however, Singer also says they must be applied with care, and he cites the example of AChE from bovine erythrocyte membranes which can be extracted by NaCl (1 mol/l) in the absence of divalent cations thus suggesting the possibility that AChE is a peripheral protein. Although he explains this by saying that membrane fragments enriched with AChE are probably released, the danger remains that liberal applying of criteria can lead to misinterpretation of results.

Since it is then possible to highly purify AChE by affinity chromatography and then bind it to liposomes, how could this be applied for future work ? It would be interesting to purify, for instance, erythrocyte bound AChE and try to bind this to liposomes. It might then be able to use this artificial system to study the possibility that erythrocyte AChE function is to transport K^+. (Giberman et al., 1973)

Also it would be of great value if AChE could be reconstituted in a liposome with the acetylcholine receptor, acetylcholine storage protein and ionophore in order to study such processes as ion fluxes and how they are affected by any vectorial bias in the proteins' position in

the membrane. Also the reconstitution of axonal AChE into liposomes might help to prove or disprove Nachmansohn's theory of chemical processes being involved with axonal transmission. From a purely subjective and philosophical point of view it is difficult to imagine how the electrical, processes of the neuronal axon can function without some considerable chemical activity and organisation playing an essential role.

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Properties of the Soluble and Membrane-Bound Forms of Acetylcholinesterase Present in Pig Brain

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Approximately 15% of the total acetylcholinesterase (AChE) activity of pig brain cortex can be extracted in dilute buffer solution and the properties of this »soluble« form of the enzyme have been compared with the membrane bound enzyme which was brought into solution by extraction with 1% Triton X-100 or 1 mM EDTA. The activity of the »soluble« enzyme against a range of substrates is identical to the membrane enzyme. The variation of activity with pH and substrate concentration are similar for the two physical forms of the AChE. Gradient polyacrylamide gel electrophoresis demonstrated the similarities of the »soluble« and detergent solubilized enzyme preparations. Three molecular weight species were common to both preparations: 353 000, 262 000, and 68 000 and in addition the »soluble« enzyme had a band of mol. wt. 135 000 while the Triton X-100 extract contained species of mol. wt. 181 000 and 83 000.

The membrane AChE showed a break in the Arrhenius plot with a transition temperature of 27 °C and this was abolished with detergent. In contrast the »soluble« enzyme showed no break in the Arrhenius plot suggesting the absence of associated membrane material. There are however more similarities than differences between the two physical forms of the enzyme which appear to be closely related.

INTRODUCTION

In all species so far studied, acetylcholinesterase (acetylcholine hydrolase. EC 3.1.1.7.) has been shown to be a membrane-bound enzyme[1,2] although its exact relationship with the membrane has not been fully clarified[3,4]. Most information on the pure enzyme has been obtained from *Electrophorus electricus* which is a very rich source of acetylcholinesterase (AChE). The enzyme from electric eel is relatively easy to solubilize and subsequently purify especially since the application of affinity chromatography[5]. The enzyme from mammalian brain however is tightly bound to the membrane and a number of different methods have been applied to bring the enzyme into solution. In particular, the use of the non-ionic detergent Triton X-100 has proved to be most useful[6,7] and more recently repeated extraction of brain homogenate with low ionic strength media containing EDTA has also been used[8,9]. McIntosh and Plummer[10] have compared several methods for bringing the enzyme into solution and have reported on the effect of the solubilization method on the

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number and distribution of the multiple molecular forms of acetylcholinesterase from pig brain. These workers showed that about 14% of the AChE was soluble in aqueous solution without further treatment and this present paper is concerned with comparing the properties of this »naturally soluble« part of the brain acetylcholinesterase with the 86% which is membrane bound. The effect of solubilization by detergent and also by EDTA extraction on the properties of the membrane enzyme has also been investigated.

A knowledge of the molecular forms of the soluble and membrane-bound acetylcholinesterase of brain and their relationship to each other should give a clearer insight into some of the molecular mechanisms of synaptic transmission.

MATERIALS AND METHODS

Chemicals

Reagents were obtained as follows: 5,5'-dithiobis-(2-nitrobenzoate) (DTNB), bovine serum albumin, acetylthiocholine iodide, (ATChI) acetylcholine iodide (AChI) and catalase from Sigma (London): EDTA and sucrose from Fisons (Loughborough, Leicestershire). Triton X-100 was obtained from BDH Chemicals Ltd (Poole, Dorset) and Carbowax 20 M purchased from G. T. Gurr and Co Ltd (London). The acetylcholinesterase inhibitor BW 284 C51 was obtained from Wellcome Reagents Ltd (Beckenham, Kent).

All other reagents were analytical grade and all solutions were prepared in glass distilled water redistilled from an all glass still.

Solubilization of acetylcholinesterase

All solubilization procedures were performed on fresh porcine brains. The brain cortex was excised then homogenized in a Waring blendor for 5 min at 4 °C followed by centrifugation in the cold in an M. S. E. ultracentrifuge S.S.65 or S.S.50. *Preparation of »soluble« enzyme.* A 20% w/v homogenate of the cortex was prepared in 0.03 M sodium phosphate buffer, pH = 7.0. The homogenate was then centrifuged at 100 000 g for 1 hour and the acetylcholinesterase in the supernatant taken to be the 'soluble' enzyme.

Solubilization with Triton X-100. Acetylcholinesterase was essentially solubilized by the method of Ho and Ellman[7]. The cortex was blended as above at 20% w/v, centrifuged at 100 000 *g* for 1 hour and the supernatant removed. The pellet was resuspended in the same volume of buffer containing 1% Triton X-100 w/v. The mixture was stirred for 10 minutes at room temperature and the suspension clarified by centrifuging at 100 000 *g* for 1 hour. The activity in the supernatant was taken as the Triton solubilized AChE.

Solubilization with EDTA. Extraction with EDTA was accomplished by modifying the method of Chan *et al.*[8] A 20% w/v homogenate was prepared in 0.03 M sodium phosphate buffer pH = 7.0 and centrifuged at 100 000 g for 1 hour. The pellet was resuspended to the original volume in a solution containing 0.03 M phosphate buffer and 10^{-3} M EDTA. This suspension was stirred for 2 hours at 4 °C, recentrifuged at 100 000 g for 1 hour and the supernatant removed. Two more extractions of the homogenate were carried out and the 3 supernatants combined and concentrated with Carbowax.

Other methods of solubilization. Other methods used including extraction with organic solvent, enzyme treatment and ultrasonication were caried out as previously described.[10]

Assay of acetylcholinesterase

pH-stat. Most of the assays of AChE were carried out at 30 °C using a pH-stat (Radiometer, Copenhagen, Denmark). The reaction mixture consisted of NaCl (0.15 M), $MgCl_2$ (1.3 mM), acetylcholine iodide (1 mM) and enzyme preparation (0.3—1.0 ml) made up to a final volume of 8.0 ml. The pH was maintained at 7.9 by the automatic

titration of 20 mM NaOH to neutralize the H^+ released from the hydrolysis of the substrate. The spontaneous H^+ release was measured for 5 min before the addition of the substrate and any appropriate correction made. Enzyme activities were expressed as μmoles/min/mg protein.

Colorimetric method. For the measurement of a large number of samples, the colorimetric method of Ellman *et al.*[11] was used to assay AChE. All spectrophotometric measurements were made at 30 °C in a Perkin-Elmer 124 spectrophotometer or Pye Unicam S.P. 800 spectrophotometer. The reaction mixture was composed of 2.83 ml of 0.1 M sodium phosphate buffer, pH = 8.0, 0.05 ml of enzyme preparation, 0.1 ml of 5,5'-dithiobis-(2-nitrobenzoate) and 0.02 ml of acetylthiocholine iodide added in that order. The hydrolysis of acetylthiocholine produces thiocholine which reacts with DTNB to give 5-thio-2-nitrobenzoate which has an extinction maximum at 412 nm.

Protein

The method of Lowry *et al.*[12] was used with crystalline bovine serum albumin as standard.

Gradient polyacrylamide gel electrophoresis

Electrophoresis was carried out on a concave gradient of polyacrylamide (4—24%) and the gels stained for AChE activity as previously described[10].

Sucrose-gradient centrifugation

Linear sucrose gradients were prepared and centrifuged according to the method of Martin and Ames[13]. The centrifuge tubes contained 5—20% w/v linear sucrose gradients (19 ml) on a cushion of 60% sucrose (2 ml). The gradients were stored at 4 °C for 5 hours then 0.5 ml of enzyme preparation together with a catalase marker was carefully overlaid onto the sucrose. The tubes were then centrifuged at 100 000 *g* for 17 hours at 4 °C in a 3×25 ml swing out rotor. Fractions (0.5 ml) were then taken from the centrifuge tubes using an M. S. E. tube piercer and assayed for AChE activity using the Ellman method.

Arrhenius plots

The activity of the enzyme preparation (v) was determined over a range of substrate concentration (s) up to 1 mM acetylcholine iodide. The maximum enzyme activity (V_{max}) was then determined from these results by plotting s/v against s or $1/v$ against $1/s$. The temperature used ranged from 5 °C to 50 °C over which the enzyme was found to be quite stable during the time of assay. Log V_{max} was then plotted against the reciprocal of the absolute temperature.

RESULTS

Solubilization of acetylcholinesterase

Pig brain cerebral cortex contains a »naturally soluble« form of AChE and this was clearly shown when a 20% brain homogenate in dilute buffer was centrifuged for 1 hour at 100 000 *g*. Under these conditions, 15% of the AChE activity of the homogenate was recovered in the supernatant. This is referred to as the »soluble« or »naturally soluble« form of the enzyme.

Treatment with detergents was found to be the most efficient way of bringing the rest of the AChE into solution and Triton X-100 at a concentration of 1% w/v solubilized about 65% of the activity and an increase in the detergent concentration to 2% made only a marginal improvement in the amount of enzyme brought into solution. Previous reports had shown that

Triton X-100 with high salt concentration solubilized almost all of the AChE present in human erythrocytes[14] and this was shown to be the case for the brain enzyme (Fig. 1). However, low concentrations of KCl suppressed the solubilization by detergent and concentrations above 0.6 M caused the separation of lipid material which was difficult to remove. A standard procedure was therefore adopted of using only 1% Triton X-100 with no KCl present. Treatment with lysolecithin was also very efficient giving 85% of the enzyme in the soluble form but the method could not be adopted on a large scale because of the expense involved.

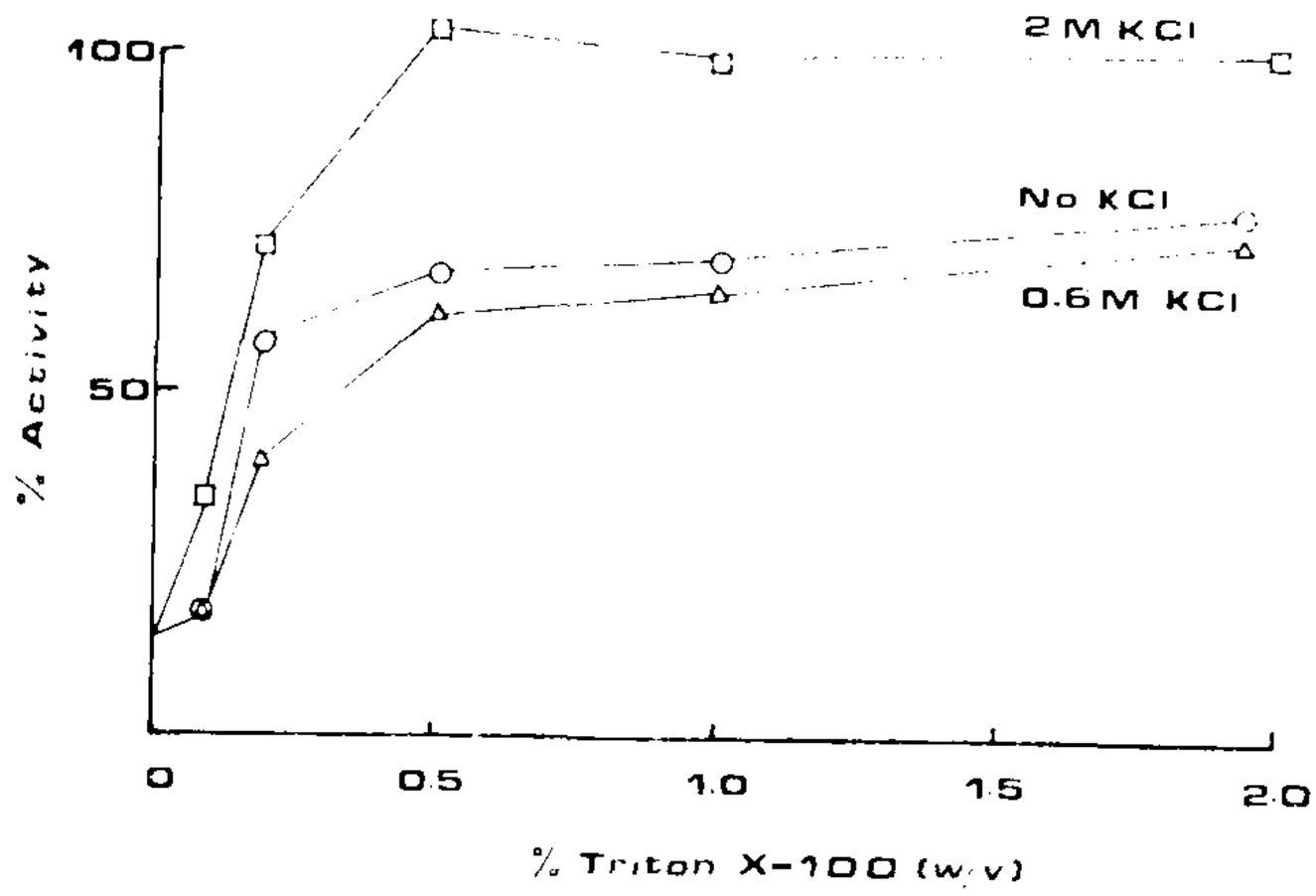

Fig. 1. Solubilization of brain acetylcholinesterase with Triton X-100 and KCl. The activity in the supernatant is plotted on the ordinate.

The other methods examined briefly were relatively ineffective at bringing the enzyme into solution and with some treatments considerable loss of activity was observed (Fig. 2).

Kinetic studies on soluble and membrane acetylcholinesterase

The standard conditions for assay were predetermined on samples of brain homogenate and enzyme solubilized with Triton X-100. Maximum activity was observed over the pH range 7.9 to 8.5 for both the membrane and solubilized preparations. Non-enzymic hydrolysis of AChI was zero up to pH = 8 but started to become significiant above this point, so all assays were carried out at pH = 7.9.

Optimum activity was obtained at 1 mM substrate concentration for the homogenate and 2 mM for the Triton X-100 extract (Fig. 3). This led to the adoption of 1 mM for all standard assays. Both preparations showed inhibition by high substrate concentrations but the solubilized enzyme was more sensitive to inhibition giving only 65% of the maximum activity at 10 mM substrate concentration compared with 85% for the homogenate. The other difference observed was in the Michaelis constants but this was only small. The solubilized enzyme had a K_m of 69 μM and the membrane enzyme gave a slightly higher value of 80 μM. The 'naturally soluble' form however had a higher K_m of 220 μM.

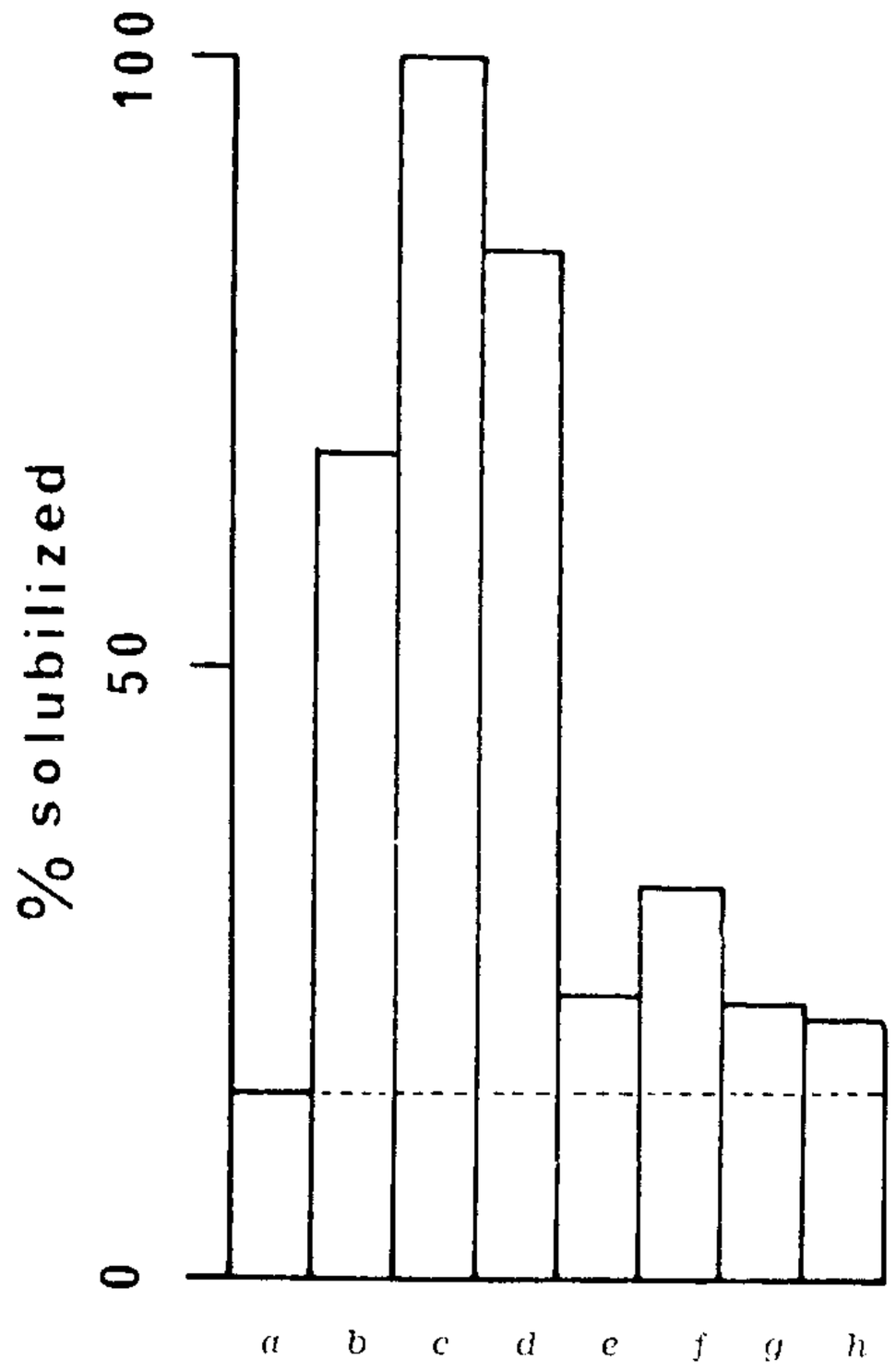

Fig. 2. Solubilization of the acetylcholinesterase of pig brain cortex.
a) Dilute buffer extract; b) 1% Triton X-100; c) 1% Triton X-100 1 M KCl; d) Lysolecithin, 12 mm; e) Butanol extract; f) EDTA, 1 mM; g) Nagarse; h) Ultrasonication.

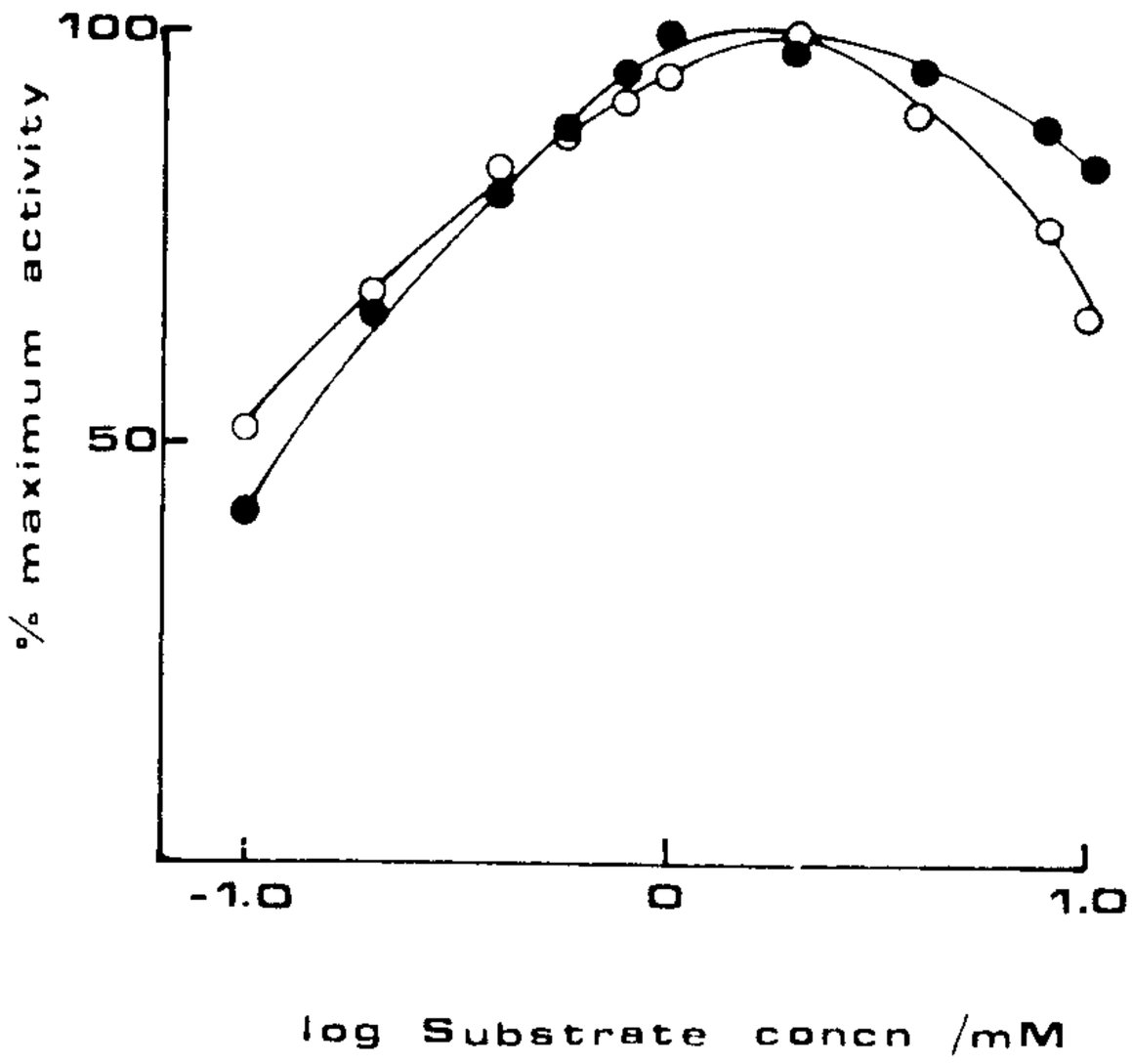

Fig. 3. The substrate concentration dependence of (●) membrane bound and (○) Triton X-100 solubilized acetylcholinesterase of pig brain.

The specificity of the »naturally soluble« AChE and the Triton X-100 extracted enzyme were found to be identical using a wide range of substrates at optimum concentration (Table I).

TABLE I.

Hydrolysis of various choline esters by brain acetylcholinesterase

Substrate	Activity as % of acetylcholine iodide	
	Soluble enzyme	1% Triton extract
Acetylcholine iodide	100	100
Propionylcholine iodide	83	84
Butyrylcholine iodide	0	0
Acetylthiocholine iodide	149	146
Acetyl-β-methylcholine bromide	20	22
Tributyrin	4	5

Multiple forms of acetylcholinesterase

Electrophoresis of the enzyme preparations on a gradient of polyacrylamide showed that the AChE exists in a number of molecular forms of differing molecular weight (Fig. 4). Assuming that these molecules are spherical, then the molecular weights obtained for the »soluble« enzyme after calibration of the gels were 340 000, 260 000, 135 000 and 68 000. The Triton-solubilized enzyme had three bands in common with the 'soluble' enzyme with mol. wt. values of 365 000 and 264 000 and 68 000. In addition to these species, a band of mol. wt. 181 000 and a faint band of 83 000 were observed. Extraction with EDTA again gave one band whose mol. wt. (250 000) was similar to those found in the other preparations. However in this case, lower mol. wt. zones

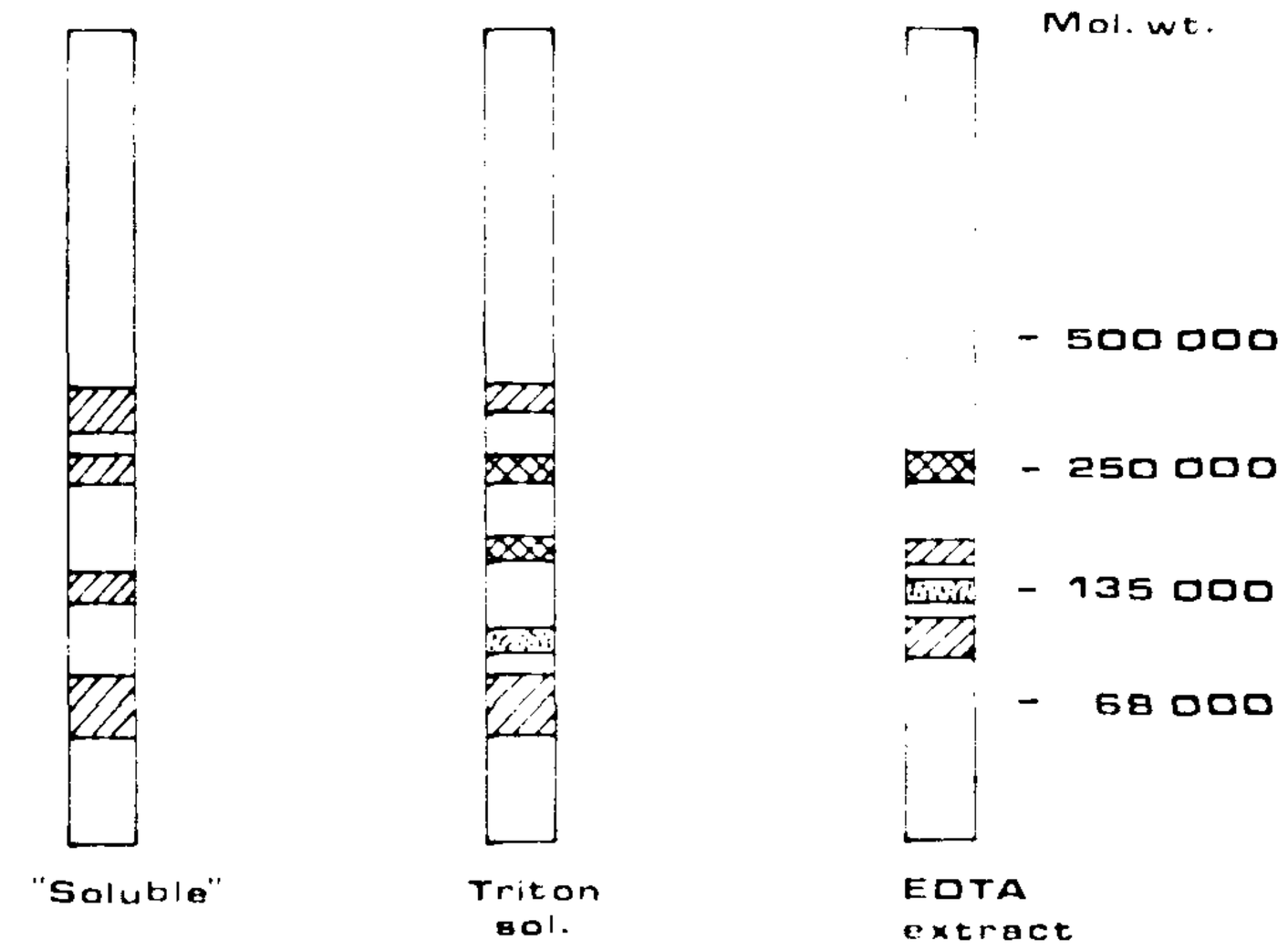

Fig. 4. Electrophoresis of soluble and solubilized acetylcholinesterase on a gradient of polyacrylamide.

were observed corresponding to mol. wt. of 160 000 and 84 000 together with a faint zone of mol. wt. 124 000.

The one feature in common is the presence of a species whose mol. wt. is in the region of 250 000 and this was the major species found after sedimentation on a sucrose gradient. The sedimentation constant lay in the region 11 to 12 S and in the case of the detergent solubilized enzyme this was the only species detected and was unaffected by high ionic strength (Fig. 5).

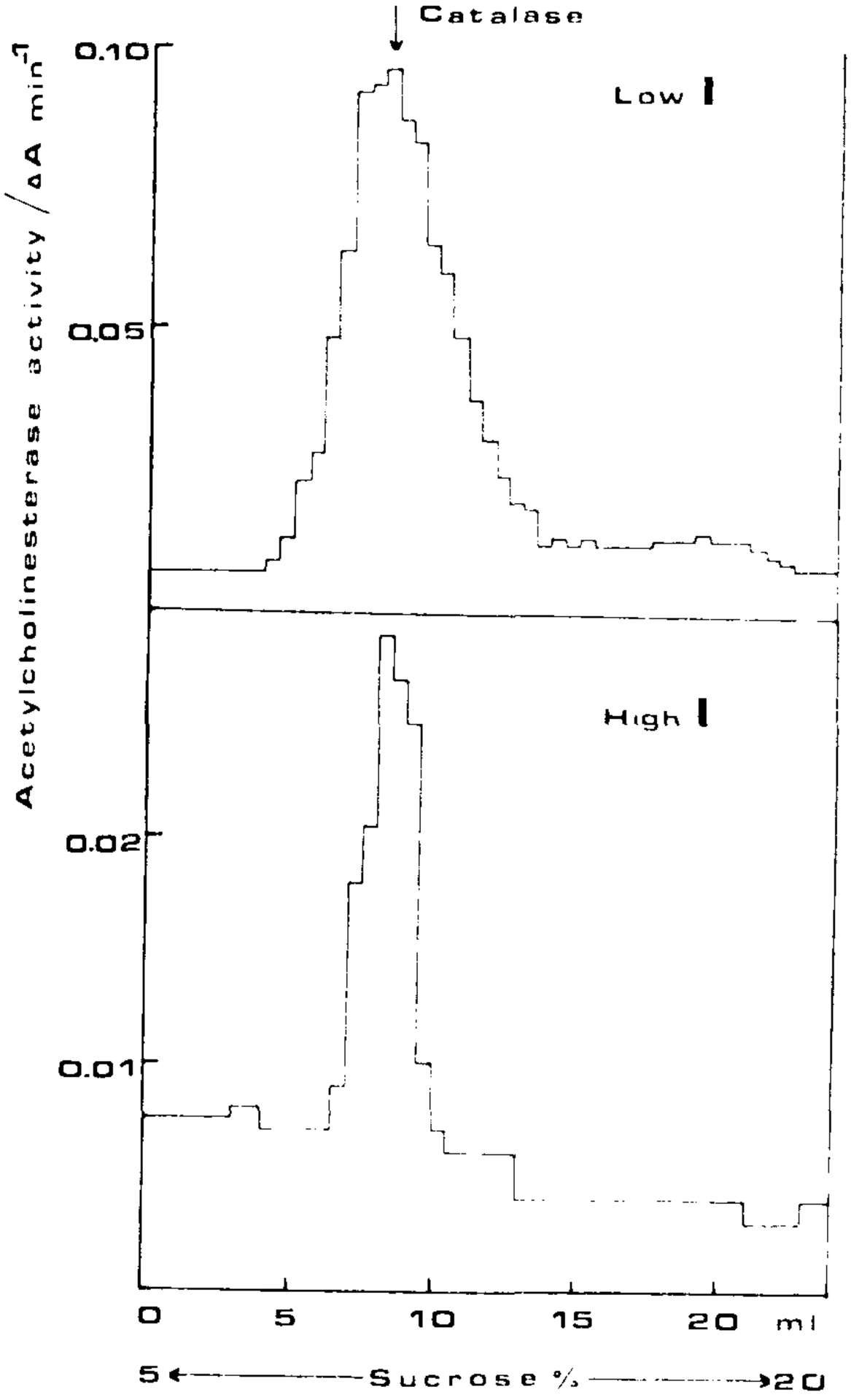

Fig. 5. Sucrose density gradient centrifugation of Triton X-100 soluble acetylcholinesterase.

Ageing of this preparation resulted in a slight loss in activity and a slight broadening of the peak but no change in the value of the sedimentation constant. Smaller peaks of higher mol. wt. were observed for the »naturally soluble« and EDTA soluble AChE and these had sedimentation constants of 15 S and 19 S approximately equivalent to mol. wt. 360 000 and 525 000. During gradient centrifugation, no molecular species less than 11.4 Sv were observed in contrast to the results obtained on electrophoresis.

Arrhenius plots

The Arrhenius plot of the membrane-bound enzyme shows two linear regions with a break at 27 °C, the transition temperature (Fig. 6a). This is often found with membrane associated enzymes and is abolished with detergent due to the loss of the membrane structure[15]. In the case of brain AChE, treatment with Triton X-100 abolishes the break and a linear plot is obtained as expected for a membrane associated enzyme (Fig. 6b). The »naturally soluble« enzyme also shows a linear plot with no break (Fig. 6c) so is probably a genuinely soluble form of the enzyme and not associated with small particles of membrane.

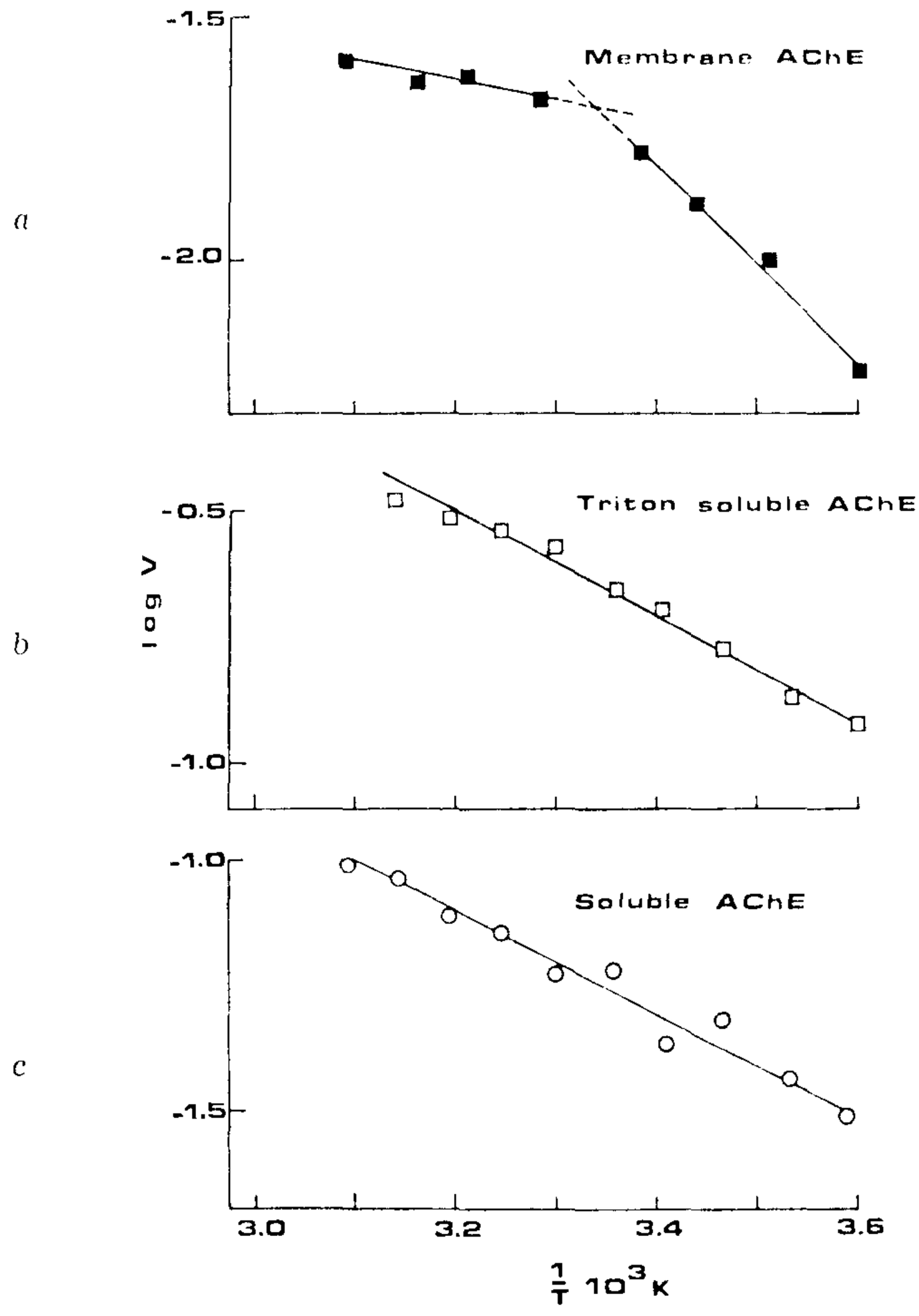

Fig. 6. Arrhenius plots of brain acetylcholinesterase. V is the maximum activity in nmol $min^{-1} mg^{-1}_{protein}$.

The energies of activation calculated from the Arrhenius plots are given in Table II. The lowest value of 8.3 kJ mol is found in the case of the membrane enzyme above the transition temperature. Solubilization with Triton X-100 increases this value to 20 kJ mol very similar to the value

TABLE II.

Activation energies of membrane and soluble brain acetylcholinesterase

	E/kJ mol^{-1}		
Membrane suspension	8.3	&	39
Aqueous extract	19		
Triton extract	20		
Human erythrocyte ghosts	2.2	&	22

found for the »naturally soluble« form of the enzyme of 19 kJ/mol. Below the transition temperature, the activation energy of the membrane preparation increases steeply to 39 kJ/mol.

DISCUSSION

The operational criterion of solubility used was if the AChE remained in the supernatant following centrifugation at 100 000 *g* for 1 hour. Using this standard, 15% of the total brain AChE was found to be soluble following homogenization of the brain with dilute buffer, a value similar to that reported by Bajger and Žižkovský[16].

Further solubilization was obtained after extraction of brain homogenate with 1% Triton X-100 although the yield of 65% was lower than that of other authors for the extraction of rat brain[7,17]. The combination of detergent and high salt concentration increased the amount of enzyme solubilized (Fig. 1) which could mean that both hydrophobic and electrostatic bonds are involved in binding the enzyme to the membrane. Another possibility is that the KCl weakens electrostatic bonds which bind membrane proteins that shield some of the AChE from the action of the detergent. In the case of human erythrocyte AChE there is some evidence from electron microscopy for this latter suggestion[14]. Unfortunately the method could not be used routinely because of the problems of removing lipid which salted out at KCl concentrations greater than 0.6 M. Lysolecithin was a good solubilizing agent but too expensive to be used routinely and of the other methods used only EDTA extraction proved effective although the yield of soluble enzyme was only 45% after three extractions. This method was used for some experiments since it is arguable that it is less traumatic than detergent treatment. None of the other methods investigated were examined further due to the small amount of AChE solubilized.

The pH-activity profiles and substrate velocity curves (Fig. 3) of the membrane AChE were altered only slightly on solubilization with Triton X-100 and the optimum values obtained were similar to brain AChE from other species[6,8]. The specificity of the solubilized enzyme was that a specific AChE and was identical to that shown by the »naturally soluble« enzyme suggesting a close relationship between the two preparations. Solubilization with detergent made little difference to the K_m value of the homogenate (69—80 μM) which was similar to that obtained for other species[6,8]. The »naturally soluble« enzyme however did have a lower enzyme-substrate affinity with a K_m of 220 μM.

Electrophoresis on a polyacrylamide gradient showed the presence of several molecular forms of AChE of different molecular weights. The membrane solubilized preparation with detergent showed a similar pattern to the naturally soluble enzyme with some small differences (Fig. 4). The EDTA extract had no molecular weights greater than 240 000 and had some low mol. wt. species which did not coincide with bands observed in the other preparations. The one feature in common to all three preparations was the presence of a molecular form with a molecular weight close to 250 000 a value obtained for AChE purified from electric eel.[18,19] Sedimentation of the Triton soluble enzyme on a sucrose gradient gave a single peak of 11.4 S (Fig. 5) and this species was also the dominant one in the naturally soluble and EDTA soluble enzymes although in these latter cases some higher molecular weight species were also detected of 15 S and 19 S. Purification of AChE from the electric eel often leads to a molecule with a sedimentation coefficient of about 11 S and this form appears to be a degradation product of larger molecules of 14 S and 18 S present in fresh tissue[20,21]. No species were found with mol. wt. lower than 250 000 during sedimentation studies although smaller enzyme molecules were detected after electrophoresis (Fig. 4). One possible explanation is that during electrophoresis molecules involved in aggregating the subunits are removed.

The Arrhenius plots of the membrane AChE clearly showed a distinct break at 27 °C. There are many reasons for the existence of such a break[22] but in the case of membrane bound enzymes it is likely to be due to a change in the physical state of the lipids from a mobile to a gel-like form[15]. This seems likely since treatment with detergent which destroys the membrane structure also abolished the break in the Arrhenius plot. The »naturally soluble« enzyme showed no break in the Arrhenius plot which suggests that this form of the enzyme is not associated with lipid material in the form of micromembranes but appears to be a genuinely soluble form of AChE. The activation energy of the membrane enzyme is quite low at 8.3 kJ mol with an approximate 5-fold increase below 27 °C. The 'naturally soluble' enzyme had an activation energy of 20 kJ/mol and the Triton X-100 soluble one was very similar to this with 19 kJ/mol. Acetylcholinesterase associated with the membrane therefore appears to be a more efficient enzyme than the soluble or solubilized form due to the lower energy barrier to be overcome. The kinetic properties are however very similar as previously discussed and if the Triton X-100 soluble enzyme is similar to the membrane enzyme then the differences in the multiple molecular forms of the »naturally soluble« and Triton soluble AChE are reelatively small. The two physical forms of the enzyme appear to be similar and it is therefore possible that the two are closely related *in vivo* and this is under investigation.

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DISCUSSION

M. E. Eldefrawi:

Have you considered the use of white *vs.* grey matter in an attempt to distinguish axonal from synaptic enzyme?

D. T. Plummer:

No. All our studies have been carried out on the pig brain cortex. It is something to consider for future experiments.

I. Silman:

Is pseudocholinesterase usually not present in brain tissue?

D. T. Plummer:

As far as I am aware some pseudocholinesterase activity is usually found in brain and the zero activity of pig brain homogenate with butyrylcholine is surprising.

W. N. Aldridge:

In your studies on the membrane bound enzyme you obtain the low apparent energies of activation at the higher temperature range. Would you like to comment on the fact that this value is so much lower than that for the enzyme in solution. Does it have any significance for studies in which we wish to make physiological derivations?

D. T. Plummer:

The low activition energy of acetylcholinesterase in pig brain has also been observed in our laboratory for the human erythrocyte enzyme. This presumably means that the enzyme is more »efficient« when attached to the membrane compared with the soluble enzyme and the detergent solubilized acetylcholinesterase. In some ways kinetic studies on a solubilized enzyme may give a false impression of the behaviour of the enzyme *in vivo*. This is especially true if membrane material is attached to the enzyme and measurements are made on the preparation below the transition temperature.

E. Heilbronn:

I am worried about the physiological significance of all the different molecular species of acetylcholinesterase and of soluble acetylcholinesterase. The latter may simply mirror regular membrane turnover. To what extent are the former artefacts of preparation?

D. T. Plummer:

The molecular weights of the various species do appear to be related suggesting the existence of oligomers, but how they are associated with the membrane *in vivo* is something we would all like to know. Similar molecular weight species of acetylcholinesterase are found using a wide range of solubilization procedures so artefacts induced this way do not seem to occur.

P. W. Taylor:

Would it not seem more likely that the discontinuity in the Arrhenius plots represent a local structural change in the membrane rather than a true phase transition of the integral membrane? While sharp transitions are observed with artificial bilayers with homogenous phospholipid composition, transitions occurring over a broad temperature range would be the likely situation for membranes of heterogeneous composition or those containing associated proteins.

D. T. Plummer:

I agree, the discontinuity in the Arrhenius plots must represent a phase transition in the membrane in the region of the acetylcholinesterase since structural changes away from the enzyme would be expected to have little or no effect on the activity of the enzyme.

G. Hollunger:

We have found that if extraction of the brain acetylcholinesterase by water is performed in the presence of DEAE-Sephadex so that the released enzyme is immediately adsorbed almost all of it appears in the 80 000 molecular weight form. This enzyme no longer aggregates (*J. Neurochem.* **20** (1972) 82). I think that this indicates that the enzyme is released from the membrane in the low molecular weight form and that an aggregating factor is stuck to the gel when the enzyme is eluted. Could the higher molecular weight forms in such a case be some sort of artefacts?

D. T. Plummer:

I would agree that there is an enzymatically active species of M. W. about 80 000 in mammalian brain but whether this is the form in which acetylcholinesterase exists *in vivo* is not clear. The interpretation that the higher molecular weight oligomers are artefacts arising by aggregation of the 80 000 unit is of course possible, although we have found no change in the electrophoretic pattern on ageing of our preparation. Another possibility of course is that a number of oligomers exist *in vivo* and that treatment with DEAE-Sephadex removes some aggregating factor so that only the subunits are found during your method of preparation. In our laboratory we have tried using DEAE-Sephadex, but this has no effect on our electrophoretic pattern. I believe that we were not following your method exactly so this will be repeated. It must not be forgotten, however, that your work was with calf brain and our own with pig brain cortex, and this could be the reason for the differences observed.

SAŽETAK

Svojstva topljive i na membranu vezane acetilkolinesteraze prisutne u mozgu svinje

D. T. Plummer, C. A. Reavill i C. H. S. McIntosh

Približno 15% od ukupne acetilkolinesterazne (AChE) aktivnosti korteksa svinjskog mozga može se ekstrahirati razrijeđenom otopinom pufera. Uspoređena su svojstva ovakvog topljivog enzima sa svojstvima enzima vezanog na membranu, koji je u topljiv oblik preveden putem ekstrakcije 1%-nom otopinom Triton X-100 ili EDTA. Oba enzimska oblika pokazuju identičnu aktivnost prema nizu iskušanih supstrata. Promjena aktivnosti s pH i koncentracijom supstrata također je slična za oba

fizička oblika acetilkolinesteraze. Sličnosti u preparacijama topljivog i detergentom otopljenog enzima, pokazane su elektroforezom na poliakrilamidnom gelu. Tri molekularne vrste s molekularnim težinama 353 000, 262 000 i 68 000 zajedničke su obim preparatima. Topljivi enzim pokazivao je također prisutnost vrste s molekularnom težinom 135 000, a ekstrakt Tritona X-100 još i vrsta s molekularnim težinama 181 000 i 83 000.

Acetilkolinesteraza vezana na membranu pokazuje prijelom na Arrheniusovom dijagramu pri temperaturi od 27 °C, no te pojave nema nakon ekstrakcije detergentom. Topljivi enzim ne pokazuje prijelom u Arrheniusovu dijagramu, što upućuje na odsutnost tvari vezanih na membranu. Budući da ima više sličnosti nego li razlika, može se zaključiti da su ta dva fizička oblika acetilkolinesteraze međusobno vrlo usko povezana.

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